Maternal diet matters

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The molecular impact of dietary fat on the offspring's immunologic, metabolic and pulmonary health

Sven Rouschop

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**The molecular impact of dietary fat on the offspring's
immunologic, metabolic and pulmonary health**

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The molecular impact of dietary fat on the offspring's immunologic, metabolic and pulmonary health

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ter verkrijging van de graad van doctor aan de Universiteit Maastricht
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Chapter 1

General introduction

Fetal programming

A fetus is highly vulnerable during its development and maternal exposures through lifestyle, diet and environment during pregnancy can greatly determine the health of the offspring at birth and later in life. For instance, smoking during pregnancy increases the risk of adverse outcomes in the child, such as fetal growth restriction, spontaneous abortion, preterm birth, and sudden infant death syndrome [1]. More specifically, offspring health is influenced by maternal diet as well. Alcohol consumption during gestation, for example, is associated with miscarriage, preterm labor, intrauterine growth restriction, and stillbirth [2]. Similarly, consumption of raw meat may result in infection with the parasite *Toxoplasma gondii*, which can lead to miscarriage, mental retardation, microcephaly, hydrocephalus, and seizures [3]. Besides, offspring health can be affected by a lack of specific nutrients as well. In the early 1990's, it was recognized that a maternal folate deficiency is linked to the incidence of neural tube defects in offspring [4]. To reduce the incidence of these fetal malformations, the Dutch Health Council started recommending women who plan to get pregnant to have a daily intake of 400 µg folic acid, starting from four weeks before conception, until eight weeks after conception.

Next to these consequences on the child's health *in utero* or directly after birth, maternal diet can have long-term effects on offspring health as well, a concept that is known as fetal programming. This idea of fetal programming was clearly shown by the Dutch famine studies, which demonstrated that malnutrition during gestation is associated with detrimental health effects in offspring later in life [5]. During the Dutch famine, which lasted from November 1944 till April 1945, official rations varied from 400 to 1000 calories per day. This caloric restriction was imposed on a previously well-nourished population and was lifted abruptly after the liberation of the Netherlands in May 1945, thereby creating a very specific and narrow window of malnourishment. Observational studies showed that exposure to this famine during gestation was associated with various adverse health effects in the offspring at an adult age, such as increased BMI and adiposity [6,7], decreased glucose tolerance [8,9], an atherogenic lipid profile [10,11], hypertension and increased blood pressure response to stress [12,13], and a higher prevalence, as well as an earlier onset of coronary heart disease [14,15].

Dietary fat

One specific dietary component that has been associated extensively with offspring health, is dietary fat. Fat is one of the three macronutrients, next to carbohydrates and proteins. In addition to providing energy, fats function as structural building blocks of cell membranes, they carry fat-soluble vitamins, such as vitamin A, D, E and K, and they are precursors of

hormones and other bioactive molecules. Different types of dietary fat exist, such as phospholipids, sterols and free fatty acids. However, the majority of dietary fats are triglycerides, which are composed of a glycerol backbone attached to three fatty acids (Figure 1). These fatty acids consist of a carboxylic group and a long carbon chain, and can be classified based on their length and saturation. Most of the naturally occurring fatty acids have an even number of carbon atoms and vary in length from four to 28 carbons per chain. Saturation refers to the number of double bonds in the carbon chain. Fatty acids can be saturated (no double bonds), monounsaturated (one double bond), and polyunsaturated (more than one double bond). In fatty acid nomenclature, the length and saturation of a fatty acid are indicated as x:yn-z, with x indicating the number of carbon atoms, y indicating the number of double bonds, and z indicating the carbon center at which the first double bond is located, starting counting at the methyl end of the chain. For example, the fatty acid 18:2n-6 has an eighteen-carbon chain, with two double bonds, of which the first is located at the sixth carbon, counted from the methyl end of the chain (Figure 2).

Figure 1. Schematic drawing of a triglyceride.

Figure 2. Schematic drawing of the fatty acid 18:2n-6 (linoleic acid).

Essential fatty acids

Although many fatty acids can be synthesized *de novo* in the liver, some fatty acids cannot, meaning that they have to be ingested through food. These so-called essential fatty acids comprise two specific classes of fatty acids: n-6 polyunsaturated fatty acids (PUFAs) and n-3 PUFAs. The n-6 PUFA with the simplest structure is linoleic acid (LA), which is abundant in nuts and seeds of plants like safflower and sunflower. LA can be further elongated and desaturated into more complex derivatives, such as arachidonic acid (AA). In addition, AA is abundant in foods such as meat and eggs. For n-3 PUFAs, the simplest fatty acid is α -linolenic acid (ALA), which can be found in seeds of plants like chia and flax. Similar to the conversion of LA into AA, ALA can be further converted into longer-chain derivatives such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Furthermore, EPA and DHA are abundant in fatty fish like salmon, herring and menhaden. Both n-6 and n-3 PUFAs have critical functions during fetal growth, including the development of the central nervous system and the retina [16]. Next to this, n-6 and n-3 PUFAs fulfill significant roles in the regulation of inflammatory responses [17]. These responses, however, differ slightly, with n-6 PUFAs being more pro-inflammatory, whereas n-3 PUFAs are considered to be more anti-inflammatory [17].

Atopy

Maternal consumption of a diet with a low n-3:n-6 PUFAs ratio has been associated with offspring atopy, corresponding with the pro- and anti-inflammatory properties of n-6 and n-3 PUFAs, respectively. Atopy is the predisposition to produce an exaggerated immunoglobulin E (IgE) response to allergens that would otherwise be harmless [18]. Different clinical manifestations of atopy are asthma, allergic rhinitis, atopic dermatitis and food allergy. Although differences exist in these manifestations and their exact pathologies, they share common characteristics such as exacerbations triggered by exposure to an allergen, a Th2-skewed immune response and inflammation [19]. Despite growing knowledge of atopic diseases, their prevalence has increased over the past few decades [20-22]. Concurrent with this growing prevalence of atopic diseases, consumption of pro-inflammatory n-6 PUFAs has increased, whereas consumption of anti-inflammatory n-3 PUFAs has decreased, resulting in the previously formulated hypothesis by Black and Sharpe that the increasing prevalence of atopic diseases may be explained by changes in the n-6 PUFA:n-3 PUFA ratio of the diet [23]. Indeed, observational studies demonstrated that maternal intake of n-3 PUFAs such as ALA, EPA and DHA during pregnancy was inversely associated with risk of asthma and asthma-related symptoms in children later in life [24-26]. Moreover, human intervention studies showed that maternal supplementation with n-

3 PUFAs during gestation is able to reduce the risk of asthma, asthma-related symptoms, and having asthma medication prescribed [27-29].

Pulmonary health

As well as the relationship between asthma in offspring and the specific fatty acid composition of the maternal diet, associations have been found between maternal high-fat diet and pulmonary health in the offspring. For example, both maternal obesity and weight gain during pregnancy are associated with elevated risk of childhood asthma and wheezing [30,31]. Similarly, feeding a high-fat diet to female rats and mice during gestation and lactation induces airway hyperresponsiveness and pulmonary inflammation, and increases susceptibility to viral respiratory infection in offspring [32,33]. Furthermore, exposure to a perinatal high-fat diet exacerbates ozone-induced responses on lung function, injury and inflammation in rat offspring [34,35]. It is important to study the underlying mechanisms of these associations and find clues for primary prevention, as the prevalence of inflammatory lung conditions is increasing in the population. According to the World Health Organization (WHO), chronic obstructive pulmonary disease (COPD) and lower respiratory infections were in the top ten global causes of death, as well as in the top ten global causes burden of disease in 2019 [36].

Metabolism

Next to offspring pulmonary health, maternal high-fat diet has consequences for offspring metabolic health, such as the risk of developing non-alcoholic fatty liver disease (NAFLD). NAFLD is the accumulation of lipids in the liver (hepatic steatosis) in the absence of excess alcohol consumption [37]. NAFLD represents a spectrum of diseases ranging from hepatic steatosis, through steatohepatitis (steatosis in combination with hepatic inflammation), to liver fibrosis and cirrhosis, which ultimately may lead to hepatocellular carcinoma. In normal weight people without any metabolic risk factors, prevalence of NAFLD is about 16% [38], whereas NAFLD prevalence is 43% in patients with diabetes [39] and 91% in obesity patients [40]. Obesity and type 2 diabetes are thus risk factor for developing NAFLD. Various mechanisms contribute to the pathogenic hepatic lipid accumulation in NAFLD (Figure 3) [41]. In type 2 diabetes patients, insulin resistance increases the efflux of free fatty acids from adipose tissue, which are then taken up by the liver. Similarly, excess intake of dietary lipids results in increased hepatic uptake of free fatty acids as well. These hepatic free fatty acids are subsequently converted into triglycerides and stored in the liver, thereby contributing to hepatic steatosis. In addition, insulin resistance leads to increased *de novo* lipogenesis and decreased β -oxidation of free fatty acids. Finally, decreased triglyceride export from the liver by very low-density lipoprotein (VLDL) particles contributes to hepatic

lipid accumulation as well. Several of these characteristics and risk factors of NAFLD have been associated with maternal diet: epidemiological studies have shown that obesity, type 2 diabetes and increased levels of body fat, insulin, and triglycerides occur more often in offspring with obese mothers than offspring with normal-weight mothers [42-45]. Moreover, feeding female mice a high-fat diet during gestation causes offspring to weigh more, have more fat mass and higher leptin levels [46], and induces accumulation of hepatic cholesterol and triglycerides, upregulation of *de novo* lipid synthesis and steatohepatitis in offspring [47].

Figure 3. Mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. VLDL, very low-density lipoprotein.

Fetal organ development

Fetal programming has been proposed to be facilitated by various mechanisms. Firstly, maternal dietary exposures may directly affect the development of a fetal organ, thereby influencing its function during later life. This was suggested by findings from the Dutch famine studies, which showed that the prevalence of obstructive airways disease was increased in adults exposed to famine in mid gestation, but not in adults exposed during early or late gestation [48]. Since bronchial trees grow most rapidly during mid gestation, fetal undernutrition during this developmental period may have affected the structure and physiology of the airways, leading to long-term adverse pulmonary effects [5]. Similarly, the

Dutch famine study demonstrated that microalbuminuria prevalence was higher in adults exposed to famine during mid gestation, but not early or late gestation [49]. This may be related to nephron numbers increasing rapidly during mid gestation, and thus determining nephron endowment and later-life kidney function [5].

Epigenetics

In addition to maternal diet directly affecting organ development, prenatal dietary exposures may influence offspring health through epigenetics. Epigenetics are heritable and functionally relevant changes to the genome that do not involve changes in the nucleotide sequence. Various epigenetic mechanisms have been described, such as histone modifications and non-coding RNAs, but the best-characterized epigenetic mechanism is DNA methylation. DNA methylation is the addition of a methyl group at the 5-position of a cytosine nucleotide. This covalent modification primarily occurs at cytosines directly followed by guanosine, a so-called CpG dinucleotide. DNA methylation is established by *de novo* methyltransferases, whereas demethylation occurs in a multi-step process involving ten-eleven-translocation enzymes [50]. The main way in which DNA methylation affects physiological processes is by regulating gene expression. This regulation of gene expression can be accomplished in several ways. In promoter and enhancer regions, DNA methylation of CpG-rich regions may block binding sites of DNA-binding proteins such as transcription factors [51,52]. Alternatively, methylated CpGs may function as recognition sites for transcriptional repressor protein complexes, thereby inhibiting gene expression [53,54]. Various DNA methylation-related effects have been associated with maternal diet. For instance, feeding mice a high-fat diet during gestation induced both differential DNA methylation and differential gene expression of metabolic and inflammatory genes in offspring [46,47,55]. Furthermore, the Dutch famine study showed that individuals who were prenatally exposed to famine had less DNA methylation of the Insulin-like growth factor 2 (*IGF2*) gene [56]. Given its functions as a fetal growth factor, differential methylation of *IGF2* and any related changes in gene expression may thus mediate any adverse effects of prenatal exposure to famine.

Aim and outline of this thesis

The aim of this thesis was to study the effects of maternal dietary fatty acids and high-fat diet on the molecular responses regarding metabolic, immunologic and pulmonary health. For this purpose, we performed three intervention studies in animals and one observational study in humans.

In **chapter 2**, we explored the roles of gene expression and DNA methylation as mechanisms of disturbed metabolism in mouse offspring after exposure to a perinatal high-fat diet. In this study, both parent mice received a high-fat diet or a control diet before and during gestation and throughout lactation, after which offspring were weaned onto a high-fat diet. In offspring, hepatic genome-wide gene expression was assessed, as well as hepatic DNA methylation of selected genes.

The study described in **chapter 3** examined whether a perinatal high-fat diet influences ozone-induced responses on pulmonary oxidant status and the molecular control of mitochondrial function and content in rat offspring. For this purpose, female rats were fed a high-fat diet or a control diet before and during gestation, and during lactation. At a young-adult age, offspring were acutely exposed to filtered air or ozone, after which offspring lungs were examined for markers related to oxidative stress and the regulation of mitochondrial function and content.

For **chapter 4**, we studied the effect of a maternal fish oil diet during gestation and lactation on *ex vivo* polarization of naïve CD4⁺ T cells. These CD4⁺ T cells play a major role in the pathology of atopic diseases, since their polarization is Th2-skewed in atopic individuals. In this study, dams received either a fish oil diet or a control diet during gestation and lactation. At the end of lactation, naïve CD4⁺ T cells were isolated from offspring spleen, polarized *ex vivo*, and examined for secretion and gene expression of Th-specific cytokines and transcription factors.

In **chapter 5**, we evaluated the multivariate association between maternal fatty acid status during pregnancy and offspring inflammation by analyzing data from the Maastricht Essential Fatty Acid Birth (MEFAB) cohort using canonical correlation analysis.

In **Chapter 6**, the main outcomes of the studies in this thesis are discussed in a broader perspective.

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Chapter 2

Gene expression and DNA methylation as mechanisms of disturbed metabolism in offspring after exposure to a prenatal HF diet

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Abstract

Exposure to a prenatal high-fat (HF) diet leads to an impaired metabolic phenotype in mouse offspring. The underlying mechanisms, however, are not yet fully understood. Therefore, this study investigated whether the impaired metabolic phenotype may be mediated through altered hepatic DNA methylation and gene expression. We showed that exposure to a prenatal HF diet altered the offspring's hepatic gene expression of pathways involved in lipid synthesis and uptake (SREBP), oxidative stress response (Nrf2) and cell proliferation. The downregulation of the SREBP pathway related to previously reported decreased hepatic lipid uptake and postprandial hypertriglyceridemia in the offspring exposed to the prenatal HF diet. The upregulation of the Nrf2 pathway was associated with increased oxidative stress levels in offspring livers. The prenatal HF diet also induced hypermethylation of transcription factor binding sites upstream of *Lpin1*, a gene involved in lipid metabolism. Furthermore, DNA methylation of *Lpin1* transcription factor binding sites correlated with mRNA expression of *Lpin1*. These findings suggest that the effect of a prenatal HF diet on the adult offspring's metabolic phenotype are regulated by changes in hepatic gene expression and DNA methylation.

Introduction

The parents' diet before and during pregnancy significantly determines their offspring's risk of developing diseases later in life [1-7]. Observational studies have shown that maternal consumption of a high-fat (HF) diet and obesity during pregnancy and lactation predisposes offspring to developing various metabolic disorders, such as obesity and type 2 diabetes, and cardiovascular diseases [8-11]. In mice, a maternal HF diet leads to increased body weight and fat mass and decreased energy expenditure in offspring [12,13]. Similarly, a paternal HF diet before conception is able to increase the offspring's bodyweight and fat mass, and to impair its lipid and glucose metabolism [14,15]. In addition, both maternal and paternal HF diet have been shown to induce non-alcoholic fatty liver disease in mouse offspring, indicated by increased liver size, excessive accumulation of cholesterol and triglycerides (TGs) and eventually hepatic inflammation [13,14]. Moreover, reports studying the influence of both paternal and maternal diet showed that the combination of these diets has an additive effect on mouse offspring health, leading to more prominent negative effects in offspring compared to only a paternal or maternal HF diet [14,16].

Although the effects of a prenatal HF diet on the offspring phenotype have been studied thoroughly, it is less known through which mechanisms these long lasting effects are transmitted. The responsible molecular mechanisms for this have been suggested to involve epigenetics, since both human and animal studies demonstrated that prenatal exposures alter DNA methylation in the offspring [12,13,17,18]. In addition, both maternal and paternal HF diet have been shown to modify gene expression, predominantly affecting pathways related to metabolism and inflammation [12,13,17]. Altering these metabolic and inflammatory pathways contributed to the impaired metabolic phenotype in offspring of HF-fed mice. Altogether, this lead to the hypothesis that a prenatal HF diet changes DNA methylation in offspring, which subsequently alters gene expression, thereby affecting the offspring's phenotype.

To test this hypothesis, we studied the effect of a combined paternal and maternal HF diet on hepatic DNA methylation and gene expression in mouse offspring. In this study, both parent mice received a HF or low-fat (LF) diet before and during pregnancy, and throughout lactation, after which offspring was weaned onto a HF diet. Feeding both parents the same diet and weaning offspring onto a HF diet better approximates the current Western situation, in which the diet is characterized by a HF content, and eating patterns are shared by individuals from the same family [19]. Only male offspring was included for analyses, since sex differences are known to occur in the response to a prenatal HF diet [12,20-23]. Previous work showed that at 12 weeks of age, the offspring displayed postprandial

hypertriglyceridemia, which related to an impaired clearance of lipids from the blood into the liver after a HF meal [24].

To study the mechanisms underlying the impaired metabolic phenotype after prenatal exposure to a HF diet, hepatic genome-wide gene expression was studied using whole transcript microarrays. In addition, hepatic DNA methylation of selected genes was assessed by bisulphite pyrosequencing. This paper shows that exposure to a prenatal HF diet alters gene expression of pathways involved in hepatic lipid synthesis and uptake, oxidative stress response and cell proliferation. Furthermore, the prenatal HF diet induces hypermethylation of transcription factor (TF) binding sites upstream of *Lipin 1* (*Lpin1*), a differentially expressed gene involved in lipid metabolism.

Materials and Methods

Study design and workflow

A schematic overview of the study design and workflow is presented in Figure 1.

Animal model

Animal experiments were approved by the Institutional Ethics Committee on Animal Welfare of Maastricht University. Mice were housed under standard conditions at 25°C with a 12-12 hour light-dark cycle with *ad libitum* access to water and feed. Specific-pathogen-free (SPF) C57Bl/6J (Charles River, Saint-Germain-Nuelles, France) parent mice were randomly assigned to either a HF diet (45% kcal fat, of which 31% saturated, 36% mono-unsaturated, and 33% polyunsaturated fatty acids; 20% kcal protein; and 35% kcal carbohydrate; Research Diets, New Brunswick, USA) or a standard chow LF diet (9% kcal fat; 33% kcal protein; and 58% kcal carbohydrate; ssniff Spezialdiäten GmbH, Soest, Germany). The diet started 6 weeks before mating and continued throughout gestation and lactation. Maternal food intake and bodyweight were measured weekly [24]. At weaning, all offspring received a HF diet, thereby creating the experimental groups HF/HF and LF/HF, with the first two letters indicating the parental diet and the last two letters indicating the offspring diet. Offspring was weaned onto a HF diet, since this experimental setup provides a better representation of the Western diet, which is predominantly characterized by a HF content. Litter size and offspring food intake were similar for both experimental groups [24]. Only male offspring was included for further experiments. Phenotypic outcomes in offspring between 12 and 28 weeks of age were previously reported [24]. To study how these effects of the prenatal HF diet were transmitted, livers were obtained from offspring at 12 weeks

(n=7 per group) and 28 (n=9 per group) weeks of age and used for subsequent measurements in gene expression, oxidative stress and DNA methylation.

Figure 1. Overview of study design and workflow. Parent mice received a HF or LF diet starting 6 weeks before mating and continuing during pregnancy and lactation. Offspring were weaned onto a HF diet, thereby creating the experimental groups LF/HF and HF/HF, with the first two letters indicating the parental diet and the last two letters indicating the offspring diet. At 12 and 28 weeks of age, offspring livers were collected and analyzed. Gene expression was assessed using microarrays and GSEA. Selected differentially expressed (DE) genes were confirmed with real-time qPCR. DNA methylation of TF binding sites upstream of differentially expressed genes was measured using bisulfite pyrosequencing. Oxidative stress was measured with the GSH/GSSG assay.

Gene expression microarray

Total RNA from offspring liver was extracted using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). RNA concentration and purity were assessed using a Nano Drop ND-1000 spectrophotometer (Isogen, IJsselstein, the Netherlands). RNA quality was measured on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands). All RNA samples had an RNA Integrity Number (RIN) >8.0. RNA (100 ng) was labelled with Whole

Transcript Sense Target Assay and hybridized to Affymetrix Mouse Gene 1.1 ST arrays (Affymetrix, Santa Clara, USA). Microarrays were analyzed using MADMAX pipeline for statistical analysis of microarray data [25]. Signal intensities were normalized with the robust multichip average (RMA) method and probes were annotated [26]. Microarray data are available through the Gene Expression Omnibus database (GSE123009).

Gene Set Enrichment Analysis

Functional data analysis of microarray data was performed with Gene Set Enrichment Analysis (GSEA) [27]. GSEA focuses on gene sets (groups of genes that share common biological function or regulation) rather than individual genes. For GSEA, annotated gene sets were used from the databases BioCarta [28], Kyoto Encyclopedia of Genes and Genomes (KEGG) [29], Reactome [30], and WikiPathways [31]. Significantly affected gene sets were selected on a false discovery rate (FDR) q-value of < 0.05 and were ranked by the normalized enrichment score (NES).

Selection candidate genes for DNA methylation analysis

Individual genes from the microarray were defined as differentially expressed when comparison of the normalized signal intensities showed a p-value <0.05 in a two-tailed paired intensity-based moderated t-statistics (IBMT) and an absolute fold-change ($|FC|$) > 1.2 [32]. Differentially expressed genes were ranked by $|FC|$ (HF/HF versus LF/HF at week 12) and filtered for involvement in lipid metabolism or inflammation. A selection was made for the top two genes that were differentially expressed at both week 12 and week 28 (*Cd163* (Cluster of differentiation 163) and *Hmgcr* (3-Hydroxy-3-methylglutaryl-CoA reductase)), and the top five genes that were differentially expressed at only week 12 and not at week 28 (*Aacs* (Acetoacetyl-CoA synthetase), *Lpin1* (Lipin 1), *Pla2g16* (Phospholipase A2 Group XVI), *Saa1* (Serum amyloid A1), and *Il1r1* (Interleukin 1 receptor, type I)).

Gene expression RT-qPCR

To confirm differential expression of candidate genes for DNA methylation, real-time quantitative (q)PCR was used. First, RNA was reverse transcribed into cDNA using iScript (Bio-Rad, Veenendaal, the Netherlands). Real-time quantitative PCR (RT-qPCR) was performed on a Bio-Rad CFX384 Real-Time PCR System using iQ SYBR Green Supermix (Bio-Rad). Primer sequences (Supplemental Table S1) were obtained from PrimerBank [33] and qPrimerDepot [34]. Housekeeping genes *Gapdh* and *Actb* were used for normalization. Relative expression levels were quantified with the $\Delta\Delta Ct$ method [35].

Selection transcription factor binding regions

Candidate genes of which differential expression was confirmed with RT-qPCR were used for subsequent DNA methylation analysis. For this purpose, CpGs were selected with the potential to regulate expression of the genes *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Saa1*, and *Il1r1*. Regions with a high density of TF binding sites were identified <2000 bp upstream of the transcription start site (TSS) of each gene (Figure 2A), by using the Gene Transcription Regulation Database (GTRD; version 17.04). GTRD is a database of TF binding sites identified by ChIP-seq experiments [36]. Genomic locations of the selected TF binding regions and CpGs within those regions are shown in Figure 2B. Methylation of CpG sites within the identified TF binding regions could potentially influence TF binding and thus regulate gene transcription. Therefore, methylation of these CpGs was measured using pyrosequencing.

Pyrosequencing

To assess DNA methylation levels of selected CpG sites, a pyrosequencing approach was used. For this, DNA from offspring liver was extracted using DNeasy Blood & Tissue Kit (Qiagen) and subjected to bisulphite conversion with EZ DNA Methylation Kit (Zymo Research, Irvine, USA) and subsequently used as template for amplification of the selected TF binding regions. PCR was performed on an Eppendorf Mastercycler nexus (Eppendorf, Nijmegen, the Netherlands), using HotStarTaq DNA polymerase (Qiagen) and dNTP mix (ThermoFisher Scientific, Eindhoven, the Netherlands). Biotin-labeled amplicons were captured with Streptavidin Sepharose beads (GE Healthcare, Vienna, Austria) and denatured and annealed to the sequencing primer using the PyroMark Q24 Vacuum Workstation (Qiagen). Amplicons were sequenced on a PyroMark Q24 (Qiagen), using PyroMark Q24 Advanced CpG Reagents (Qiagen). Methylation of each CpG was quantitatively assessed using the PyroMark Q24 Advanced software. Assays and primers for PCR and pyrosequencing (available upon request) were designed with PyroMark Assay Design software.

GSH and GSSG assay

To assess oxidative stress in offspring livers, reduced and oxidized glutathione (GSH and GSSG, respectively) were measured according to Rahman et al. [37]. In short, liver homogenate was mixed with 5-sulfosalicylic acid to prevent further oxidation of GSH to GSSG. Supernatant of this mixture was used for both GSH and GSSG assessment. GSH was measured by oxidizing it with 5,5-dithiobis-(2-nitro-benzoic acid) (DTNB) to form TNB, which is measurable with a spectrophotometer. For GSSG measurement, samples were first pre-

incubated with 2-vinylpyridine to deplete all GSH. Subsequently, samples were incubated with NADPH and glutathione reductase, which reduces GSSG into GSH, and simultaneously incubated with DTNB to quantify the amount of GSH through TNB formation. GSH and GSSG concentrations were normalized to the starting amount of liver tissue.

Figure 2. Identification of TF binding regions and selection of CpG sites for pyrosequencing. A1: Regions with a high density of TF binding sites were identified <2,000 bp upstream of the TSS of a selected gene, based on GTRD. A2: CpG sites within the selected TF binding regions were selected for DNA methylation analysis. B: Genomic positions of TF binding regions (indicated by black horizontal lines) and CpGs within these regions (indicated by vertical lines), which were selected for DNA methylation analysis. Arrows mark TSS.

Statistical analysis

For GSH, GSH/GSSG ratio, RT-qPCR, and DNA methylation, differences between groups were tested by two-tailed, independent samples Student t test. For DNA methylation, p-values were corrected for multiple testing by computing FDR q-values using the Benjamini-Hochberg procedure [38]. For GSSG, differences between groups were tested by two-tailed Mann-Whitney U test. The relation between DNA methylation level of TF binding regions and mRNA expression was described using Pearson correlation coefficient with log transformed mRNA expression levels. $P < 0.05$ or $q < 0.05$ was considered significant. All statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

Results

A prenatal HF diet alters hepatic expression of gene sets involved in lipid metabolism, oxidative stress response and cell proliferation

Previous work showed that at 12 weeks of age, HF/HF offspring displayed postprandial hypertriglyceridemia and an impaired clearance of lipids from the blood into the liver after a HF meal [24]. To study whether this impaired metabolic phenotype in HF/HF offspring was related to alterations in hepatic gene expression, a microarray was performed on offspring livers at 12 and 28 weeks of age. As a result, a total of 492 genes was found to be differentially expressed in 12 week old HF/HF offspring, compared to LF/HF offspring. Moreover, GSEA revealed that the prenatal HF diet significantly affected expression of four different pathways in offspring liver at 12 weeks of age, with two being upregulated and two downregulated (Table 1). The two downregulated pathways were related to hepatic lipid biosynthesis and uptake (i.e., *Activation of gene expression by SREBP*) and cellular movement (i.e., *Striated muscle contraction*), whereas the upregulated pathways were linked to oxidative stress response [i.e., *nuclearfactor (erythroid-derived 2)-like 2 (Nrf2) targets*] and intracellular transport (i.e., *Kinesins*).

In 28 week old offspring, expression of 534 individual genes was altered in HF/HF offspring compared to LF/HF offspring. Furthermore, the prenatal HF diet significantly affected expression of 63 pathways, of which two pathways were upregulated, whereas 61 pathways were downregulated. The two upregulated pathways were both related to Sema4D signaling. Of the downregulated pathways, however, the majority was involved in cell proliferation, having functions in mitosis, cell cycle, proteasomal degradation, Wnt signaling and Hedgehog signaling. In addition, hepatic lipid biosynthesis and uptake was still

Table 1. Pathways significantly affected by prenatal HF diet in offspring liver at 12 weeks (n=7 per group) and 28 weeks (n=9 per group) of age. For each biological function, the top three (based on normalized enrichment score (NES)) significant pathways are shown. A negative or positive NES indicates that the pathway was downregulated or upregulated, respectively, in HF/HF offspring compared to LF/HF offspring. A complete overview of all significantly affected pathways is listed in Supplemental Table S2. Pathway database: ^aWikiPathways; ^bReactome; ^cKEGG; ^dBioCarta.

Biological Function of Pathway	Pathway	NES	FDR
12 weeks			
Cellular movement	Striated muscle contraction ^a	-2.34	0.004
Lipid biosynthesis / uptake	Activation of gene expression by SREBP ^b	-2.32	0.002
Oxidative stress response	Nrf2 targets ^b	2.60	<0.000
Intracellular transport	Kinesins ^b	2.08	0.015
28 weeks			
Lipid biosynthesis / uptake	Cholesterol biosynthesis ^b	-2.29	0.002
	Activation of gene expression by SREBP ^b	-1.95	0.006
	Steroid biosynthesis ^c	-1.91	0.009
Mitochondrial translation	Mitochondrial translation initiation ^b	-2.28	0.001
	Mitochondrial translation elongation ^b	-2.28	0.001
	Mitochondrial translation termination ^b	-2.20	0.001
Antigen processing / presentation	Cross-presentation of soluble exogenous antigens (endosomes) ^b	-2.22	0.001
	Antigen processing-Cross presentation ^b	-1.77	0.028
	ER-Phagosome pathway ^b	-1.84	0.016
Mitosis / Cell cycle	Autodegradation of Cdh1 by Cdh1:APC/C ^b	-2.20	0.001
	APC/C:Cdc20 mediated degradation of mitotic proteins ^b	-2.18	0.001
	APC/C:Cdc20 mediated degradation of Securin ^b	-2.18	0.001
Proteasomal degradation	Proteasome ^c	-2.15	0.001
	Proteasome pathway ^d	-2.11	0.001
	Proteasome degradation ^a	-1.99	0.005
HIV-host interaction	Vpu mediated degradation of CD4 ^b	-1.93	0.008
	Vif-mediated degradation of APOBEC3G ^b	-1.93	0.008
Regulation of transcription and translation	Ribosome biogenesis in eukaryotes ^c	-2.00	0.005
	Eukaryotic transcription initiation ^a	-1.91	0.009
	RNA transport ^c	-1.89	0.011
Wnt signaling	Degradation of axin ^b	-1.89	0.011
	Asymmetric localization of PCP proteins ^b	-1.88	0.011
	Degradation of DVL ^b	-1.80	0.022
Hedgehog signaling	Hedgehog ligand biogenesis disease ^b	-1.80	0.022
	Degradation of GLI1 by the proteasome ^b	-1.79	0.023
	Processing defective Hh variants are degraded by the proteasome ^b	-1.78	0.027
Sema4D signaling	Sema4D in semaphorin signaling ^b	2.11	0.040
	Sema4D-induced cell migration and growth cone collapse ^b	2.06	0.038

downregulated in 28 week old HF/HF offspring, including gene sets *Activation of gene expression by SREBP*, *Cholesterol biosynthesis* and *Steroid biosynthesis*. Altogether, a prenatal HF diet altered hepatic expression of gene sets involved in lipid biosynthesis and uptake, oxidative stress response and cell proliferation.

A prenatal HF diet increases oxidative stress in offspring liver

Since the prenatal HF diet upregulated hepatic expression of the Nrf2 pathway in 12 weeks old offspring, we next examined whether livers of these mice also presented increased levels of oxidative stress, which was done by measuring GSH and GSSG. The results show that GSH levels were similar in all four groups, whereas GSSG levels were significantly increased in HF/HF offspring compared to LF/HF at 12 weeks of age (Figure 3). Consequently, the GSH/GSSG ratio was significantly decreased in HF/HF offspring compared to LF/HF at 12 weeks of age, indicating an increased exposure to oxidative stress in the livers of HF/HF mice. In addition, there was a tendency ($p = 0.06$) for decreased GSH/GSSG ratio in 28 week old compared to 12 week old LF/HF offspring, indicating that oxidative stress tended to increase over time in the control group.

A prenatal HF diet induces hypermethylation of TF binding sites upstream of *Lpin1*

To examine whether the observed effect of a prenatal HF diet on hepatic gene expression is regulated by alterations in DNA methylation, further analysis was performed on candidate genes *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Pla2g16*, *Saa1* and *Il1r1*. These genes were selected based on their involvement in lipid metabolism (*Hmgcr*, *Aacs*, *Lpin1*, and *Pla2g16*) or inflammation (*Cd163*, *Saa1*, and *Il1r1*) and their significant differential expression in the microarray. This differential expression was verified by RT-qPCR, which showed that the prenatal HF diet

Figure 3. GSH and GSSG concentrations and the ratio between GSH and GSSG concentrations in 12-week-old ($n = 7$ for LF and $n = 6$ for HF) and 28-week-old ($n = 9$ per group) offspring livers after both parent mice received either a prenatal LF or HF diet. Boxplots represents minimum, first quartile, median, second quartile, and maximum. * $P < 0.05$, based on two-tailed independent samples t-test (GSH and GSH/GSSG) or two-tailed Mann-Whitney U test (GSSG).

significantly altered hepatic mRNA expression of *Cd163* and *Hmgcr* at 12 and 28 weeks of age, whereas expression of *Aacs*, *Lpin1*, *Saa1* and *Il1r1* was only significantly changed at week 12 (Figure 4). For *Pla2g16*, differential expression could not be confirmed with RT-qPCR and it was therefore not included for further analyses.

Figure 4. Effect of prenatal HF diet on hepatic mRNA expression of *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Pla2g16*, *Saa1*, and *Il1r1* in 12-week-old ($n = 7$ per group) and 28-week-old-offspring ($n = 9$ per group) analyzed by microarray and real-time qPCR. Expression was normalized against *Actb* and *Gapdh* and presented relative to control (LF/HF) expression (controls have Log2-fold change of 0). Bars indicate mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, based on two-tailed paired intensity-based moderated t-statistics (microarray) or two-tailed independent samples t-test (real-time qPCR).

For each of the confirmed genes, methylation was measured at CpGs within TF binding sites, thereby having the potential to regulate expression of that gene. Analysis showed that at 12 weeks of age, hepatic DNA methylation of *Lpin1* TF binding region 2 (Chr12: 16,590,890 – 16,591,130 bp) was significantly increased ($\Delta=7.1\%$, $q=0.002$) in HF/HF offspring compared to LF/HF offspring (Figure 5). At 28 weeks of age, there was no longer a significant difference, with *Lpin1* region 2 DNA methylation levels of HF/HF offspring partly being restored to the level of LF/HF offspring. No significant changes were observed for *Lpin1* TF binding regions 1 and 3, nor for any TF binding region upstream of *Cd163*, *Hmgcr*, *Aacs*, *Saa1* and *Il1r1* at 12 or 28 weeks of age (Supplemental Figure S1).

Figure 5. Effect of prenatal HF diet on hepatic DNA methylation of *Lpin1* TF binding region 2 (Chr12: 16,590,890-16,591,130 bp) at 12 weeks (n = 7 per group) and 28 weeks of age (n = 9 per group). Lines and error bars indicate mean $\pm$ SEM. **FDR q-value < 0.01, based on two-tailed independent samples t-test followed by correction for multiple testing using the Benjamini-Hochberg procedure.

mRNA expression of *Lpin1* correlates with DNA methylation of *Lpin1* TF binding sites

The observed hypermethylation of *Lpin1* TF binding region 2 in 12 week old HF/HF offspring was concurrent with a decrease in mRNA expression of *Lpin1*, indicating a negative relation between DNA methylation and gene expression for this locus. Existence of a correlation, however, would provide a stronger indication for DNA methylation to be involved in regulation of gene expression. Therefore, Pearson correlation was assessed for mean DNA methylation levels of TF binding regions versus microarray expression levels of the corresponding gene. This analysis showed that at 12 weeks of age, *Lpin1* mRNA expression correlated significantly with DNA methylation of *Lpin1* TF binding region 2 ($r = -0.54$, $p < 0.05$). Furthermore, *Saa1* mRNA expression and DNA methylation of *Saa1* TF binding region 1 also correlated significantly ($r = 0.53$; $p < 0.05$). At 28 weeks of age, both these correlations were no longer significant. However, there was a significant correlation between *Lpin1* mRNA expression and DNA methylation of *Lpin1* TF binding region 3 ($r = 0.50$, $p < 0.05$) at 28 weeks of age. Interestingly, this *Lpin1* region 3 is a reported binding site for both the TFs Nrf2 and SREBP-1 (obtained from GTRD, v. 17.04).

Discussion

While the phenotypic effects of a prenatal HF diet on offspring have been studied thoroughly, it is less well known through which mechanisms these long lasting effects are transmitted. Therefore, we studied the effect of a combined paternal and maternal HF diet on hepatic DNA methylation and gene expression in adult mouse offspring. We showed that

this prenatal HF diet altered hepatic DNA methylation and gene expression of pathways involved in lipid metabolism, oxidative stress response and cell proliferation. We hypothesize that these changes underlie the previously reported postprandial hypertriglyceridemia and decreased hepatic lipid clearance in offspring exposed to a prenatal HF diet [24].

First, we showed that a prenatal HF diet consistently downregulated the gene set *Activation of gene expression by SREBP* at both 12 and 28 weeks of age. SREBP (Sterol regulatory element-binding protein) represents a group of TFs that regulate expression of genes involved in fatty acid and cholesterol synthesis and uptake [39-44]. Since SREBP targets are involved in lipid uptake, the observed downregulation of hepatic SREBP signaling in HF/HF offspring may contribute to the impaired postprandial hepatic lipid clearance, as was previously reported in these offspring mice [24]. The observed downregulation of SREBP signaling is in agreement with two previous studies which examined the effect of a maternal HF diet on offspring liver. These studies reported that SREBP gene and protein expression were diminished and that this regulated the observed decrease in mRNA expression of genes involved in fatty acid, cholesterol and steroid biosynthesis, and other SREBP-related functions [45,46]. In contrast, two other studies found an increase in mRNA expression of *Srebp1c* and some of its downstream genes involved in *de novo* lipogenesis in response to a prenatal HF diet [13,14]. Although the reasons for these opposite effects on SREBP expression in similar experimental settings remain unclear, these reports do underline the involvement of SREBP in the effects of a prenatal HF diet on offspring liver.

Next to a decreased hepatic lipid uptake induced by SREBP downregulation, alterations in the metabolic phenotype of HF/HF offspring can also be a result of an increased export of lipids from the liver into the circulation. Such an increased export may be facilitated by the upregulation of the pathway *Kinesins* in HF/HF offspring. Kinesins are motor proteins that are, amongst other processes, required for intracellular transport of TG-rich lipid droplets to the smooth endoplasmic reticulum in hepatocytes [47]. This transport enables lipid droplets to be catabolized and TGs to be available for VLDL assembly and subsequent secretion from the liver. Upregulation of these kinesin motors could therefore contribute to enhanced VLDL-mediated export of lipids from the liver to the blood, thereby adding to the previously reported trend for decreased fasted hepatic lipid content in HF/HF offspring [24].

Besides the *Kinesins* pathway, the prenatal HF diet also increased the expression of the gene set *Nrf2 targets* in 12 weeks old offspring. Nrf2 (Nuclear factor erythroid-derived 2-like 2) is a TF that regulates expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation[48-50]. To examine whether these mice also displayed

increased levels of oxidative stress, intracellular levels of oxidized and reduced glutathione were measured in offspring livers. This showed that at 12 weeks of age, oxidative stress levels were indeed significantly increased in HF/HF offspring liver compared to LF/HF. At 28 weeks of age, however, oxidized and reduced glutathione levels, as well as expression of the Nrf2 pathway, were no longer different between HF/HF and LF/HF offspring. Instead, both groups displayed elevated levels of oxidative stress, similar to the levels observed in 12 weeks old HF/HF offspring. These findings suggest that the post-weaning HF diet led to a gradual increase in oxidative stress levels in livers, irrespective of the parent's diet. If the parents were additionally exposed to a HF diet, the increased levels of oxidative stress were already reached at 12 weeks of age, thereby prolonging the time that offspring livers were exposed to oxidative stress. This link between a HF diet and oxidative stress response has been indicated before by studies showing that pharmacologic or transgenic activation of Nrf2 protects mice from the effects of a HF diet, such as obesity, insulin resistance and liver steatosis [51,52]. Furthermore, it has previously been reported that exposure to a prenatal HF diet affected not only hepatic mRNA levels [17,53], but also DNA methylation of Nrf2-related genes [17], implying that epigenetics may regulate the effects of a prenatal HF diet on oxidative stress response.

To further investigate the involvement of epigenetics in the effects of a prenatal HF diet, DNA methylation was measured at CpGs within TF binding sites of selected differentially expressed genes. We found that the prenatal HF diet hypermethylated a TF binding region upstream of *Lpin1*. In addition, DNA methylation of two *Lpin1* TF binding regions correlated significantly with *Lpin1* gene expression. The observed correlations suggest that in current study *Lpin1* mRNA expression was (at least partly) regulated by DNA methylation. The lipin 1 protein has several biological functions, depending on the tissue in which it is expressed [54-56]. In liver, lipin 1 mainly functions as a transcriptional co-activator and its knockdown leads to increased VLDL secretion and elevated plasma TGs [55]. Therefore, hypermethylation of *Lpin1* TF binding regions and subsequent downregulation of *Lpin1* expression may explain the phenotypic changes in HF/HF offspring, including increased circulatory TGs and decreased hepatic lipids.

In addition to *Lpin1*, a correlation was also observed between a TF binding region and mRNA expression for *Saa1*. *Saa1* is an acute phase protein mainly produced by hepatocytes in response to inflammation and tissue damage. Normally, the liver is able to recover from tissue damage [57-59]. In the current study, however, pathway analysis of microarray data showed that the prenatal HF diet downregulated expression of various gene sets involved in mitosis, cell cycle, proteasomal degradation, Wnt signaling and Hedgehog signaling in offspring liver. Although we do not have phenotypical data to further support this, these results are an indication that cell proliferation is reduced in HF/HF offspring, possibly leading

to impaired recovery from liver damage. These findings are in line with a previous study which reported that rat offspring of mothers fed a HF diet showed hepatic cell cycle inhibition, together with related changes in gene expression and DNA methylation [60]. Similarly, many genes involved in cell growth, differentiation, proliferation and development were both differentially expressed and differentially methylated in livers of offspring of HF diet-fed mothers [17]. Furthermore, it has been shown that exposure to HF diet not only before birth but also after birth results in decreased liver proliferation, indicated by impaired liver regeneration after partial hepatectomy in mice [61,62]. Therefore, alterations in tissue damage and cell proliferation in liver after a prenatal HF diet deserve further attention in future research.

In comparison to previous reports, our study has a few advantages. Firstly, whereas most studies only examined the effects of a prenatal HF diet at one specific offspring age, we performed measurements in offspring at 12 and 28 weeks of age. This enabled us to analyze alterations in gene expression and DNA methylation over time. Secondly, many studies only focus either on the phenotypic outcome of a prenatal HF diet or on mechanisms underlying these effects. In our experiment, previously published effects on the metabolic phenotype of offspring were now linked to alterations in gene expression, DNA methylation and oxidative stress. Finally, most studies examine the effects of a prenatal HF diet by combining a maternal HF diet with a paternal LF diet or vice versa. However, in our study both father and mother were fed the same diet to better mimic the current Western dietary situation in which family members share eating patterns [19]. In addition, combining a maternal and paternal HF diet can have additive effects on the offspring's metabolic health, resulting in more prominent health effects in offspring when compared to separate maternal or paternal HF diets [14,16].

With the current study design, we did not assess the effect of a post-weaning LF diet. All offspring was weaned onto a HF diet, since this experimental setup provides a better model for the current Western type of diet, which is characterized by a HF content. Furthermore, it has been shown that a post-weaning HF diet may aggravate the negative effects of the prenatal HF diet [15]. Another study limitation is that the analyses were limited to male offspring. Sex differences occur in response to a prenatal HF diet, with male offspring having a larger increase in bodyweight and plasma TG [23], whereas female offspring have higher blood pressure and leptin levels [20,21]. Likewise, exposure to a prenatal HF diet differently affects hepatic gene expression in male and female offspring [23]. Major contributors to these sex dimorphisms are estrogens and androgens [63,64]. In females, these effects depend on the hormonal cycle, making female mice more prone to variation in metabolic outcomes. Therefore, only male offspring were included in present study.

In summary, we showed that a combined maternal and paternal HF diet alters hepatic gene expression of pathways related to lipid synthesis and uptake, oxidative stress response, and cell proliferation. The downregulation of lipid uptake could account for the previously observed decreased postprandial hepatic lipid uptake and hypertriglyceridemia in HF/HF offspring. Furthermore, the upregulation of the Nrf2-mediated oxidative stress response was confirmed by assessment of GSH/GSSG ratios in livers of offspring. In other words, the changes in gene expression due to prenatal HF diet were mechanistically linked to phenotypic alterations. We propose that the observed effects on the offspring's metabolic phenotype and gene expression are driven by alterations in DNA methylation, as indicated by the hypermethylation of TF binding sites upstream of *Lpin1*, and the correlation between DNA methylation of *Lpin1* TF binding sites and gene expression of *Lpin1*. Society may have to invest in early life nutrition to prevent common diseases occurring in offspring at older ages. More knowledge is needed to unravel the molecular mechanisms, including DNA methylation and other epigenetic mechanisms, that underlie the negative effects of a prenatal HF diet on offspring health.

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Supplement

Table S1. Real time qPCR primers.

Gene	Primer	Sequence
<i>Cd163</i>	Forward	GGTGGACACAGAATGGTTCTTC
	Reverse	CCAGGAGCGTTAGTGACAGC
<i>Hmgcr</i>	Forward	TGTTACCGGCAACAACAAGA
	Reverse	CCGCGTTATCGTCAGGATGA
<i>Aacs</i>	Forward	GTGGAATCGTCTACTCACGCA
	Reverse	TAAAGGGCGACTCTGTCTGTTT
<i>Lpin1</i>	Forward	AAGAGACTGACAACGATCAGGA
	Reverse	TTCCCCAGAGAACCAGTGGAT
<i>Pla2g16</i>	Forward	GTCTGCTTTGACTGACAAGGC
	Reverse	GTGTACTCCTCGTCATGTTTGT
<i>Saa1</i>	Forward	TCATGTCAGTGTAGGCTCGC
	Reverse	CCAGGAGACACCAGGATGAA
<i>Il1r1</i>	Forward	GTGCTACTGGGGCTCATTTGT
	Reverse	GGAGTAAGAGGACACTTGCGAAT
<i>Gapdh</i>	Forward	TTGATGGCAACAATCTCCAC
	Reverse	CGTCCCGTAGACAAAATGGT
<i>Actb</i>	Forward	ATGGAGGGGAATACAGCCC
	Reverse	TTCTTTGCAGCTCCTTCGTT

Figure S1. Effect of prenatal HF diet on offspring hepatic DNA methylation of TF binding regions upstream of *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Saa1* and *Il1r1* at 12 weeks (n = 7 per group) and 28 weeks of age (n = 9 per group). Lines and error bars indicate mean $\pm$ SEM. **FDR q-value < 0.01.

Table S2. A complete overview of all pathways significantly affected by prenatal HF diet in offspring liver at 12 weeks (n = 7 per group) and 28 weeks (n = 9 per group) of age. A negative or positive NES indicates that the pathway was downregulated or upregulated, respectively, in HF/HF offspring compared to LF/HF offspring. Pathway source: ^aWikiPathways; ^bReactome; ^cKEGG; ^dBioCarta.

12 weeks			
Biological function of pathway	Pathway	NES	FDR
Cellular movement	Striated muscle contraction ^a	-2.34	0.004
Lipid biosynthesis / uptake	Activation of gene expression by SREBP ^b	-2.32	0.002
Oxidative stress response	Nrf2 targets ^b	2.60	<0.000
Intracellular transport	Kinesins ^b	2.08	0.015

28 weeks			
Biological function of pathway	Pathway	NES	FDR
Lipid biosynthesis / uptake	Cholesterol biosynthesis ^b	-2.29	0.002
	Activation of gene expression by SREBP ^b	-1.95	0.006
	Steroid biosynthesis ^c	-1.91	0.009
Mitochondrial translation	Mitochondrial translation initiation ^b	-2.28	0.001
	Mitochondrial translation elongation ^b	-2.28	0.001
	Mitochondrial translation termination ^b	-2.20	0.001
	Mitochondrial translation ^b	-2.20	0.001
	Cross-presentation of soluble exogenous antigens (endosomes) ^b	-2.22	0.001
Antigen processing / presentation	Antigen processing-Cross presentation ^b	-1.77	0.028
	ER-Phagosome pathway ^b	-1.84	0.016
	Autodegradation of Cdh1 by Cdh1:APC/C ^b	-2.20	0.001
Mitosis / Cell cycle	APC/C:Cdc20 mediated degradation of mitotic proteins ^b	-2.18	0.001
	APC/C:Cdc20 mediated degradation of Securin ^b	-2.18	0.001
	Activation of APC/C and APC/C:Cdc20 mediated degradation of mitotic proteins ^b	-2.16	0.001
	Cdc20:Phospho-APC/C mediated degradation of Cyclin A ^b	-2.14	0.001
	APC/C:Cdh1 mediated degradation of Cdc20 and other APC/C:Cdh1 targeted proteins in late mitosis/early G1 ^b	-2.14	0.001
	Stabilization of p53 ^b	-2.11	0.001
	Autodegradation of the E3 ubiquitin ligase COP1 ^b	-2.10	0.001
	Regulation of activated PAK-2p34 by proteasome mediated degradation ^b	-2.06	0.002
	CDK-mediated phosphorylation and removal of Cdc6 ^b	-2.01	0.004
	Regulation of apoptosis ^b	-1.99	0.005
	p53-Independent G1/S DNA damage checkpoint ^b	-1.99	0.005
	Ubiquitin Mediated Degradation of Phosphorylated Cdc25A ^b	-1.98	0.005
	P53 independent DNA damage response ^b	-1.98	0.005
	P53 dependent G1/S DNA damage checkpoint ^b	-1.98	0.005
	SCF(Skp2)-mediated degradation of p27/p21 ^b	-1.97	0.005

	Ubiquitin-dependent degradation of Cyclin D1 ^b	-1.97	0.005
	P53 dependent G1 DNA damage response ^b	-1.97	0.005
	APC/C-mediated degradation of cell cycle proteins ^b	-1.96	0.006
	Regulation of mitotic cell cycle ^b	-1.95	0.006
	Ubiquitin-dependent degradation of Cyclin D1 ^b	-1.95	0.006
	Regulation of APC/C activators between G1/S and early anaphase ^b	-1.94	0.007
	CDT1 association with the CDC6:ORC:origin complex ^b	-1.91	0.009
	Cyclin A:Cdk2-associated events at S phase entry ^b	-1.90	0.009
	G1/S DNA damage checkpoints ^b	-1.90	0.009
	Cyclin E associated events during G1/S transition ^b	-1.84	0.017
	Regulation of ornithine decarboxylase (ODC) ^b	-1.80	0.023
	SCF-beta-TrCP mediated degradation of Emi1 ^b	-1.75	0.033
	Assembly of the pre-replicative complex ^b	-1.74	0.034
Proteasomal degradation	Proteasome ^c	-2.15	0.001
	Proteasome pathway ^d	-2.11	0.001
	Proteasome degradation ^a	-1.99	0.005
HIV-host interaction	Vpu mediated degradation of CD4 ^b	-1.93	0.008
	Vif-mediated degradation of APOBEC3G ^b	-1.93	0.008
Regulation of transcription and translation	Ribosome biogenesis in eukaryotes ^c	-2.00	0.005
	Eukaryotic transcription initiation ^a	-1.91	0.009
	RNA transport ^c	-1.89	0.011
	AUF1 (hnRNP D0) binds and destabilizes mRNA ^b	-1.85	0.015
	Ribosome ^c	-1.83	0.018
	Basal transcription factors ^c	-1.75	0.033
	Regulation of mRNA stability by proteins that bind AU-rich elements ^b	-1.75	0.033
Wnt signaling	Degradation of axin ^b	-1.89	0.011
	Asymmetric localization of PCP proteins ^b	-1.88	0.011
	Degradation of DVL ^b	-1.80	0.022
Hedgehog signaling	Hedgehog ligand biogenesis disease ^b	-1.80	0.022
	Degradation of GLI1 by the proteasome ^b	-1.79	0.023
	Processing defective Hh variants are degraded by the proteasome ^b	-1.78	0.027
	Hedgehog ligand biogenesis ^b	-1.76	0.033
	Degradation of GLI2 by the proteasome ^b	-1.75	0.033
	GLI3 is processed to GLI3R by the proteasome ^b	-1.72	0.040
Sema4D signaling	Sema4D in semaphorin signaling ^b	2.11	0.040
	Sema4D-induced cell migration and growth cone collapse ^b	2.06	0.038

Chapter 3

Perinatal high-fat diet influences ozone-induced responses on pulmonary oxidant status and the molecular control of mitophagy in female rat offspring

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Abstract

Previous research has shown that a perinatal obesogenic, high-fat diet (HFD) is able to exacerbate ozone-induced adverse effects on lung function, injury, and inflammation in offspring, and it has been suggested that mitochondrial dysfunction is implicated herein. The aim of this study was to investigate whether a perinatal obesogenic HFD affects ozone-induced changes in offspring pulmonary oxidant status and the molecular control of mitochondrial function. For this purpose, female Long-Evans rats were fed a control diet or HFD before and during gestation, and during lactation, after which the offspring were acutely exposed to filtered air or ozone at a young-adult age (forty days). Directly following this exposure, the offspring lungs were examined for markers related to oxidative stress; oxidative phosphorylation; and mitochondrial fusion, fission, biogenesis, and mitophagy. Acute ozone exposure significantly increased pulmonary oxidant status and upregulated the molecular machinery that controls receptor-mediated mitophagy. In female offspring, a perinatal HFD exacerbated these responses, whereas in male offspring, responses were similar for both diet groups. The expression of the genes and proteins involved in oxidative phosphorylation and mitochondrial biogenesis, fusion, and fission was not affected by ozone exposure or perinatal HFD. These findings suggest that a perinatal HFD influences ozone-induced responses on pulmonary oxidant status and the molecular control of mitophagy in female rat offspring.

Introduction

The prevalence of obesity has nearly doubled since 1980 [2], resulting in 52% of the European Union's (EU) adult population being overweight and 16% being obese [3]. Maternal obesity during pregnancy negatively affects the offspring's health, predisposing the offspring to obesity, type 2 diabetes, and cardiovascular diseases [4-7]. Furthermore, prenatal obesity affects lung health, with children from obese mothers being more susceptible to wheezing and developing asthma [8,9]. Similarly, a prenatal obesogenic high-fat diet (HFD) in rats and mice has been shown to induce airway hyperresponsiveness, airway inflammation, and increased susceptibility to respiratory infections [10,11]. Because of these adverse pulmonary effects, the offspring from obese mothers may be more susceptible to environmental exposures affecting the health of the lung, such as ozone.

Ozone is a secondary air pollutant that originates from photochemical reactions between precursor pollutants, such as nitrogen oxides (NO_x) and volatile organic compounds (VOCs), under the influence of sunlight. Upon inhalation, ozone has various adverse effects on the lungs, such as decreased lung function, increased lung inflammation, airway hyperresponsiveness, and increased pulmonary vascular permeability [12-16]. To protect the population against these pulmonary responses and other harmful effects induced by ozone, a target value of 0.06 ppm was set by the EU for air quality standards. Nevertheless, in 2018, 34% of the EU's urban population was exposed to ozone concentrations sufficiently high to cause harmful pulmonary effects [17].

Despite the increasing obesity prevalence and a large part of the urban population being exposed to harmful ozone levels, little research has been done on the combined effects of prenatal obesity and air pollution on the lungs of offspring. So far, two studies have shown that ozone-induced detrimental effects on lung function, injury, and inflammation in rat offspring were more profound in the offspring from dams that were fed a HFD diet during gestation [18,19]. Furthermore, a study deploying a systemic metabolomics approach demonstrated that a perinatal HFD exacerbated ozone-induced effects on lipid, protein, and energy metabolism, which suggested an impairment of mitochondrial function [1]. To elucidate whether mitochondria and changes in the molecular machinery that control the mitochondrial content and function are involved in the effect of maternal obesity on ozone susceptibility in offspring, we performed an experiment in which female rats were fed a HFD before and during gestation, and during lactation, after which the offspring were exposed to ozone at a young-adult age (forty days). Directly following this exposure, the offspring lungs were examined for markers related to mitochondrial content and function, such as oxidative stress; oxidative phosphorylation; and mitochondrial fusion, fission, biogenesis, and mitophagy. In this paper, we show that acute ozone exposure increases oxidative status

and upregulates the molecular control of receptor-mediated mitophagy in the lungs of young-adult rats, and that these responses are exacerbated in female offspring from dams fed a perinatal HFD.

Materials and methods

Animals, diet, and breeding

The animal experiments were approved by the U.S. Environmental Protection Agency's (EPA) Institutional Animal Care and Use Committee (LAPR#18-06-002). All of the animals were SPF-free, housed under 12 h/12 h light/dark cycles, and had ad libitum access to food and water. Initial feeding and breeding procedures were performed by Charles River Laboratories (Raleigh, NC, USA). Thirty-day-old female Long-Evans rats were fed either a control diet (CD; 10 kcal% fat; TD.08806, Harlan Laboratories, Teklad Custom Research Diets, Madison, WI, USA) or a high-fat diet (HFD; 60 kcal% fat, TD.06414) throughout the experiment (Figure 1). Females were bred on postnatal day (PND) 72 and shipped to the close-by EPA animal facility on gestational day (GD) 1. From each litter ($n = 10$ per diet group), two pups per sex were randomly selected and allocated to either the control or ozone exposure group (1 pup/sex/litter/exposure; $n = 10$ pups per sex/exposure/diet group). The offspring were first weaned onto the maternal diet (CD or HFD) on PND 21, and switched to CD on PND 29.

Figure 7. Experimental design. PND—postnatal day; GD—gestational day; CD—control diet; HFD—high-fat diet; O₃—ozone.

Ozone exposure

On PND 40, the offspring were exposed to either filtered air or ozone (0.8 ppm) for 5 h. The animals were exposed in Rochester style “Hinner’s” chambers. Ozone was generated from oxygen using a silent arc discharge generator (OREC, Phoenix, AZ, USA) and was transported to the exposure chambers. All of the exposures occurred between 7 am and 11 am so as to avoid diurnal variation in biological endpoints. The mean ($\pm$ standard error of the mean (SEM)) air temperature, relative humidity, and ozone concentration in the exposure chambers were 22.47 ± 0.14 °C, $52.72 \pm 0.22\%$, and 0.0 ± 0.0 ppm, respectively, for filtered air exposure, and 23.24 ± 0.12 °C, $51.09 \pm 0.22\%$, and 0.7999 ± 0.0016 ppm, respectively, for ozone exposure.

Euthanasia and sample collection

Within two hours after filtered air or ozone exposure, the animals were euthanized with an overdose of sodium pentobarbital (>200 mg/kg; Fatal Plus, Vortech Pharmaceuticals, LTD., Dearborn, MI, USA). The unlavaged left lung was snap frozen and stored at -80 °C until further assessment of the glutathione levels, total antioxidant capacity, lipid peroxidation, gene expression, and protein expression.

Glutathione status

The glutathione status was assessed by measuring reduced and oxidized glutathione (GSH and GSSG, respectively). To measure GSSG, the samples were first depleted of GSH by incubating the lung homogenates with 3% (v/v) 2-vinylpyridine (Sigma-Aldrich, Saint Louis, MO, USA) and 6% (v/v) ethanol for 1 h at room temperature. To measure the GSH + GSSG, lung homogenates were prepared in 1.3% (w/v) 5-sulfosalicylic acid (Sigma-Aldrich) so as to prevent the oxidation of GSH. Reactions were started by adding 100 μ L reagent, containing 0.3 mM 5,5-dithiobis(2-nitro-benzoic acid) (Sigma-Aldrich) and 0.4 mM NADPH (Sigma-Aldrich), and 50 μ L glutathione reductase (4 U/mL) to 50 μ L prepared lung homogenates. Absorbance was recorded at 412 nm for 3 min at 20 s intervals. GSSG and GSH + GSSG concentrations were determined using the reaction rate and standard curves. The GSH and GSSG concentrations were normalized to the starting amount of lung tissue. The GSSG/GSH ratio was calculated as a measure for the glutathione status.

Total antioxidant capacity

The total antioxidant capacity was determined using the trolox equivalent antioxidant capacity (TEAC) assay. Lung homogenates were deproteinized by incubating samples in 5%

trichloric acid for 5 min on ice, followed by centrifugation at 15,000 rpm for 5 min. To assess the antioxidant capacity, supernatants were incubated with a radical solution, containing 5 mM 2,2 azino-bis-3 ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich), 20 μ M H₂O₂, and 20 mU/mL horseradish peroxidase (Sigma-Aldrich) at 37 °C for 5 min, and the absorbance was measured at 734 nm. TEAC was calculated by normalizing the absorbances to an antioxidant capacity of 1 μ M Trolox, and was further normalized to the starting amount of lung tissue.

Lipid peroxidation

The lipid peroxidation was assessed by measuring the malondialdehyde (MDA) levels using the thiobarbituric acid reactive substances (TBARS) assay. Lung homogenates were incubated with a reagent containing 12 mM 2-thiobarbituric acid (Merck-Millipore, Burlington, MA, USA), 0.01% (w/v) EDTA (Merck-Millipore), 320 mM H₃PO₄, and 6.81 mM butylated hydroxytoluene (Sigma-Aldrich) at 100 °C for 1 h. The reaction was stopped by putting samples on ice, 0.5 vol. 1-butanol was added, and the samples were vortexed and centrifuged at 12,000 rpm for 3 min. Fluorescence was measured in supernatant at $\lambda_{exc.} = 530$ nm and $\lambda_{em.} = 560$ nm. The MDA levels were quantified using a standard curve and were normalized to the starting amount of lung tissue.

Gene expression

The total RNA was extracted from the offspring lung using TRIzol Reagent (Thermo Fischer Scientific, Waltham, MA, USA). Then, 400 ng RNA was reverse transcribed into the cDNA with iScript (Bio-Rad, Hercules, CA, USA). Real-time qPCR was performed with 4.4 μ L of 1:50 diluted cDNA, target-specific primers (Table S1), SensiMix SYBR and Fluorescein Kit (Meridian Bioscience, Cincinnati, OH, USA), and a LightCycler 480 (Roche, Basel, Switzerland). The gene expression levels were quantified with LinRegPCR software version 2014.0 [20] and normalized using the geometric mean of the expression levels of the reference genes *Tuba1b*, *Rpl13a*, and *Ppia*. Samples with non-specific melt curves or sub-optimal amplification (detected as unstable baseline or no plateau in the amplification curve by LinRegPCR software) were excluded from the analysis, leading to $n = 9$ –10 per group for all of the examined genes.

Protein expression

The offspring lung lysates were prepared in an immunoprecipitation buffer (Table S2). The protein concentrations of the lysates were measured with Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA). Lysates were boiled in a 1 $\times$ Laemmli buffer

(Table S3) and 10 µg protein per sample was separated on 12% Criterion XT Bis-Tris Protein Gel (Bio-Rad) and blotted onto 0.45 µm nitrocellulose membranes (Bio-Rad). The membranes were stained for the total protein with PonceauS (Sigma-Aldrich, Saint Louis, MO, USA) and imaged on the Amersham Imager 600 (GE Healthcare, Chicago, IL, USA). For the antigen-specific expression levels, the membranes were probed with primary antibodies (Table S4) and HRP-conjugated secondary antibodies (Table S5), incubated with SuperSignal West PICO or Femto Chemiluminescent Substrate (Thermo Fisher Scientific), and imaged on the Amersham Imager 600. The total protein levels and antigen-specific expression were quantified with ImageJ version 1.53e [21]. The antigen-specific expression was normalized for the total protein levels. Given the large number of animals, the samples were analyzed on four different gels/blots. To prevent bias from this, an equal number of samples from each experimental group was analyzed on each gel/blot. In addition, between-gel/blot variation was removed using factor correction [22], which was confirmed using principle component analysis. For some offspring animals, insufficient tissue was left over for the protein analysis after all of the previous measurements, leading to $n = 7$ for ozone-exposed CD females and $n = 9$ – 10 for all of the other experimental groups.

Statistical analysis

All data were stratified for sex and analyzed using two-way ANOVA, with diet and exposure as factors. First, it was checked whether both factors had a significant interaction. If not, the interaction term was removed from the analysis. Next, it was tested whether one or both factors had a significant effect on the outcome. For those factors with a significant effect, Holm–Sidak post-hoc test was used to compare the means. In addition to these primary analyses, associations were assessed between the glutathione status, total antioxidant capacity, lipid peroxidation, gene expression, and protein expression vs. previously published outcomes from the same experiment, such as markers for lung injury and inflammation [1]. These associations were determined using Pearson’s product–moment correlation after stratification for sex and diet. Given the large number of correlations that were tested, p -values were corrected for multiple testing by computing the false discovery rate q -values using the Benjamini–Hochberg procedure [23]. The results were graphically presented as boxplots, with boxes representing the first quartile, median, and third quartile, and whiskers representing the minimum and maximum values within 1.5 times the interquartile range (IQR) below the first quartile or above the third quartile, respectively, and with points representing the values outside the limits of the whiskers. $p < 0.05$ or $q < 0.05$ was considered significant. All of the statistical analyses were performed in R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Figure 2. Acute ozone exposure increased pulmonary oxidant status in female offspring from high-fat diet (HFD) dams and all male offspring. (a) Reduced glutathione (GSH), (b) oxidized glutathione (GSSG) and (c) GSSG/GSH ratio, (d) *Sod2* mRNA expression, and (e) SOD2 protein expression were assessed within two hours after exposure to filtered air or ozone in the lungs of female and male offspring from dams fed a perinatal control diet (CD) or HFD. Data are presented as boxplots. Statistical analysis by two-way ANOVA with a Holm–Sidak post-hoc test. $n = 10$ per group (a–c), $n = 9–10$ per group (d), or $n = 7–10$ per group (e). * $p < 0.05$, ** $p < 0.01$. O₃—ozone.

Results

Acute ozone exposure increases pulmonary oxidant status

We previously showed that the perinatal HFD compared with the CD increased the body weight and fat percentage of dams throughout gestation, as well as of the offspring at postnatal day (PND) 37 [1,19]. Furthermore, acute ozone exposure was shown to cause pulmonary protein leakage and lung cell injury in both female and male offspring, and induced neutrophilic inflammation in female offspring [1]. We next studied whether these ozone-induced pulmonary responses in offspring were associated with alterations in oxidative stress markers and whether these responses were affected by maternal diet. For the antioxidant glutathione, reduced (GSH) levels were not affected (Figure 2a), but ozone exposure did significantly increase the levels of oxidized glutathione (GSSG), and therefore the GSSG/GSH ratio, compared with air exposure in female offspring from HFD dams (Figure 2b,c). Ozone exposure also significantly upregulated mRNA expression of the mitochondrial antioxidant superoxide dismutase 2 (*Sod2*) in female offspring from HFD dams, and male offspring from both CD and HFD dams (Figure 2d). In addition, *Sod2* mRNA levels correlated significantly with previously published [1] pulmonary vascular leakage markers albumin and total protein in bronchoalveolar lavage fluid (BALF) in female and male offspring from HFD dams (Table 1). Altogether, these data suggest that acute ozone exposure increased the pulmonary oxidant status in female offspring from HFD dams and all male offspring.

Table 1. Lung *Sod2* mRNA levels correlated significantly with bronchoalveolar lavage fluid (BALF) vascular leakage markers albumin and total protein in female and male offspring from high-fat diet (HFD) dams. All of the markers were assessed within two hours after exposure to filtered air or ozone in the lungs or BALF from female and male offspring from dams fed a perinatal control diet (CD) or HFD. BALF albumin and total protein data were previously published [1]. Only significant correlations ($q < 0.05$) are shown in this table. Statistical analysis by Pearson's product-moment correlation after stratification for sex and diet. False discovery rate q -values were computed from p -values using the Benjamini–Hochberg procedure to correct for multiple testing.

Correlation	Stratum	r	q-Value
<i>Sod2</i> mRNA vs. BALF albumin	Female CD	0.42	0.624
	Female HFD	0.78	0.014
	Male CD	0.59	0.462
	Male HFD	0.82	0.003
<i>Sod2</i> mRNA vs. BALF total protein	Female CD	0.49	0.550
	Female HFD	0.77	0.014
	Male CD	0.58	0.462
	Male HFD	0.80	0.004

Not all oxidative stress markers, however, were affected by ozone exposure. Although the *Sod2* mRNA levels were upregulated in most offspring groups, this did not lead to increased SOD2 protein levels in any of the offspring groups (Figure 2e). Furthermore, no differences were observed in the Trolox equivalent antioxidant capacity (TEAC) and levels of the lipid peroxidation product malondialdehyde (MDA) (Figure 3a,b), indicating that acute ozone exposure and perinatal HFD did not alter the total antioxidant capacity or induce oxidative damage to lipids, respectively, in the offspring lungs. The mRNA expression of cytoplasmic antioxidants catalase (*Cat*) and superoxide dismutase 1 (*Sod1*) were also not altered by ozone exposure or perinatal HFD (Figure 3c,d).

Figure 3. Acute ozone exposure and perinatal high-fat diet (HFD) did not alter the pulmonary (a) total antioxidant capacity, (b) lipid peroxidation, or expression of cytoplasmic antioxidant genes (c) *Cat* and (d) *Sod1*. All of the markers were assessed within two hours after exposure to filtered air or ozone in the lungs of female and male offspring from dams fed a perinatal control diet (CD) or HFD. Data are presented as boxplots. Statistical analysis by two-way ANOVA with a Holm–Sidak post-hoc test. $n = 10$ per group (a,b) or $n = 9–10$ group (c,d). O₃—ozone.

Figure 4. Acute ozone exposure altered the molecular control of receptor-mediated mitophagy rather than ubiquitin-mediated mitophagy. Receptor-mediated mitophagy: (a) *Bnip3* mRNA expression, (b) *BNIP3* protein expression, (c) *Bnip3l* mRNA expression, (d) and *BNIP3L* protein expression. Ubiquitin-mediated mitophagy: (e) *Pink1* mRNA expression. All of the markers were assessed within two hours after exposure to filtered air or ozone in lungs of female and male offspring from dams fed a perinatal control diet (CD) or high-fat diet (HFD). Data are presented as boxplots. Statistical analysis by two-way ANOVA with a Holm–Sidak post-hoc test. $n = 9$ –10 per group (a,c,e) or $n = 7$ –10 per group (b,d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. O₃—ozone.

Acute ozone exposure alters the abundance of key constituents involved in receptor-mediated mitophagy

As acute ozone exposure upregulated the mRNA expression of the mitochondrial antioxidant *Sod2*, but not of the cytoplasmic antioxidants *Cat* and *Sod1*, we next examined whether the molecular control of the mitochondrial content in the offspring lungs was affected. Considering that mitochondria are not only a major source of reactive oxygen species (ROS), but can also be damaged by it, we first assessed the gene and protein expression of key regulators of mitophagy. As a result, the mRNA expression of B-cell lymphoma 2/adenovirus E1B 19 kDa protein-interacting protein 3 (*Bnip3*), a mitophagy receptor [24], was significantly upregulated after ozone exposure in female offspring from CD dams and all male offspring (Figure 4a). Similarly, the BNIP3 protein expression was significantly increased after ozone exposure in the male offspring from CD and HFD dams (Figure 4b). The mRNA and protein expression of B-cell lymphoma 2/adenovirus E1B 19 kDa protein-interacting protein 3-like (*Bnip3l*), a mitophagy receptor with a close homology to *Bnip3* [25], were not affected by ozone exposure or perinatal HFD (Figure 4c,d). Likewise, no differences were observed in the mRNA expression of phosphatase and tensin homolog-induced kinase 1 (*Pink1*; Figure 4e), a gene essential for ubiquitin-mediated mitophagy [26]. These results suggest that acute ozone exposure induced changes in the abundance of the molecular machinery controlling the receptor-mediated mitophagy in the offspring lungs rather than ubiquitin-mediated mitophagy in the female offspring from CD dams and all male offspring.

Acute ozone exposure and perinatal HFD do not alter the expression of the oxidative phosphorylation machinery

As ozone exposure seemed to alter the molecular regulation of mitophagy, we further assessed the abundance of key constituents of the complexes involved in oxidative phosphorylation. However, no differences were observed in the mRNA and protein levels of subunits of oxidative phosphorylation complexes I to V (Figure 5a–g), demonstrating that acute ozone exposure or perinatal HFD did not affect the expression of the oxidative phosphorylation machinery in the offspring lungs.

Figure 5 (continued on next page).

Figure 5. Perinatal high-fat diet (HFD) or acute ozone exposure did not change the pulmonary expression of oxidative phosphorylation machinery subunits. mRNA expression of (a) *Ndufb3* (complex I), (b) *Sdhb* (complex II), (c) *Cyc1* (complex III), and (d) *Cox4i1* (complex IV), and the protein expression of (e) SDHB (complex II), (f) UQCR2 (complex III), and (g) ATP5A (complex V) were assessed within two hours after exposure to filtered air or ozone in the lungs of female and male offspring from dams fed a perinatal control diet (CD) or HFD. Data are presented as boxplots. Statistical analysis by two-way ANOVA with Holm–Sidak post-hoc test. $n = 9\text{--}10$ per group (a–d) or $n = 7\text{--}10$ per group (e–g). O₃—ozone.

Figure 6. Perinatal high-fat diet (HFD) or acute ozone exposure did not affect the molecular regulation of mitochondrial biogenesis. (a) *Ppargc1a*, (b) *Ppargc1b*, and (c) *Tfam* mRNA levels were measured within two hours after exposure to filtered air or ozone in the lungs of female and male offspring from dams fed a perinatal control diet (CD) or HFD. Data are presented as boxplots. Statistical analysis by two-way ANOVA with a Holm–Sidak post-hoc test. $n = 9\text{--}10$ per group. O₃—ozone.

Figure 7. Acute ozone exposure and perinatal high-fat diet (HFD) did not alter the pulmonary molecular regulation of mitochondrial fission or fusion. Expression of fission genes (a) *Drp1* and (b) *Fis1*, and fusion genes (c) *Mfn1*, (d) *Mfn2*, and (e) *Opa1* was measured within two hours after exposure to filtered air or ozone in the lungs of female and male offspring from dams fed a perinatal control diet (CD) or HFD. Data are presented as boxplots. Statistical analysis by two-way ANOVA with a Holm–Sidak post-hoc test. $n = 9–10$ per group. O₃—ozone.

Acute ozone exposure and perinatal HFD do not affect the abundance of key regulators of mitochondrial biogenesis, fission or fusion in lung tissue

To further test whether the molecular control of the mitochondrial content was affected, we measured the expression of the master regulators of mitochondrial biogenesis peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*) and peroxisome proliferator-activated receptor gamma coactivator 1-beta (*Ppargc1b*), and of a key regulator of mitochondrial gene expression transcription factor a, mitochondrial (*Tfam*). However, the mRNA expression of these genes was not altered (Figure 6), indicating that mitochondrial biogenesis was not affected by acute ozone exposure or perinatal HFD. Next to the mitophagy and biogenesis, mitochondrial function and content is also regulated through the events of fission and fusion. To examine whether mitochondrial fission and fusion were altered by ozone exposure or perinatal HFD, the gene expression of fission mediators dynamin-related protein 1 (*Drp1*) and mitochondrial fission protein 1 (*Fis1*) was examined, as well as the gene expression of fusion mediators mitofusin-1 (*Mfn1*), mitofusin-2 (*Mfn2*), and optic atrophy 1 (*Opa1*). No differences were observed, however, in the mRNA levels of any of these genes (Figure 7a–e). Altogether, these data suggest that the processes of mitochondrial biogenesis, fission, and fusion were likely not affected in the offspring lungs by acute ozone exposure or perinatal HFD.

Discussion

Previous research has shown that a perinatal HFD is able to exacerbate ozone-induced pulmonary responses, and has suggested mitochondrial dysfunction to be involved in this effect [1,18,19]. Whether or not disturbances in the molecular regulation of the mitochondrial content and function are associated with this, however, is unknown. We therefore examined the lungs of offspring exposed to a perinatal HFD and ozone for molecular markers related to oxidative stress and the regulation of the mitochondrial content. We showed that acute ozone exposure increased pulmonary oxidant status and upregulated the abundance of the key constituents involved in the molecular control of receptor-mediated mitophagy. For female offspring, perinatal HFD aggravated these ozone responses, whereas for male offspring, the responses were similar in both diet groups. Abundances of mRNAs and proteins involved in oxidative phosphorylation and mitochondrial biogenesis, fusion, and fission were not affected by ozone exposure or perinatal HFD.

First, we showed that acute ozone exposure upregulated the mRNA expression of the antioxidant gene *Sod2* in all offspring, except for the females from CD dams. In addition, the

glutathione oxidation status (GSSG/GSH ratio) was increased in female offspring from HFD females. Collectively, these results suggest that ozone exposure increased the pulmonary oxidant status, and that perinatal HFD aggravated these responses in female offspring only. The upregulation of *Sod2* in response to both acute and chronic ozone exposure had previously been shown in other animal studies [27,28]. In addition, both *Sod2* and glutathione appear to play an important role in protecting the mitochondria against ozone-induced oxidative stress. For instance, *Sod2* overexpression and glutathione supplementation reduced p62 knockout-induced mitochondrial ROS in mouse embryonic fibroblasts [29]. Furthermore, increasing the intracellular GSH pool in yeast was shown to reduce fasting-induced mitophagy [30,31], but not other non-selective forms of autophagy [30]. In addition, genetic linkage analyses have linked the *Sod2* and glutathione peroxidase (*Gpx1*) genes to differential ozone susceptibility in mice [32,33]. Next to these specific effects on *Sod2* and glutathione, ozone exposure has also previously been shown to induce pulmonary oxidative stress in general. In both acute and chronic exposure studies in mice, ozone exposure induced pulmonary oxidative stress [34-36]. The administration of MitoQ or MitoTEMPO, which are a mitochondrion-specific antioxidant and an inhibitor of mitochondrial ROS, respectively, reversed not only the ozone-induced oxidative stress, but also restored other ozone-induced pulmonary responses, such as lung inflammation and airway hyperresponsiveness [34-36], suggesting that oxidative stress is an important mediator of other, more phenotypical pulmonary ozone responses. This may explain the correlations we found between the *Sod2* mRNA levels and BALF albumin and total protein levels, which are both markers for lung damage.

In contrast with *Sod2* expression, acute ozone exposure and perinatal HFD did not affect the mRNA expression of *Cat* and *Sod1*. Similar to *Sod2*, *Sod1* is responsible for converting superoxide to hydrogen peroxide and oxygen, whereas *Cat* catalyzes the decomposition of hydrogen peroxide into water and oxygen. *Cat* and *Sod1* exert their functions mainly in the cytoplasm and peroxisomes, respectively, while *Sod2* is located in the mitochondrial matrix. Given the different effects of ozone and perinatal HFD on expression of *Sod2* vs *Sod1* and *Cat*, and taking into account the different localizations of these enzymes, our results may suggest that the oxidative response to ozone exposure was focused at the mitochondria rather than other cellular locations.

The MDA levels were not affected by perinatal HFD or ozone exposure, indicating that neither of these treatments induced oxidative damage to lipids in the offspring lungs. The absence of lipid peroxidation could imply that cells were sufficiently protected by cellular antioxidant systems against the ozone-induced increase in oxidant status. Alternatively, oxidative damage may have occurred at a later time point after ozone exposure, as was previously observed in other studies. Xu et al., for instance, reported elevated MDA levels

in BALF after acute ozone exposure in mice [36]. However this outcome was assessed 24 h after exposure, providing the oxidative damage a larger period of time to develop than the two hours used in our study. In addition, Xu et al. exposed the animals to an ozone concentration of 2.5 ppm, which is three times higher than our 0.8 ppm. In a study by Li et al., six weeks of ozone exposure did not change the MDA levels in BALF [28]. However, after another six weeks, during which ozone exposure ceased, the MDA levels were increased, indicating a delayed oxidant response. Possibly, lipid peroxidation would have been increased in our study as well, if the pulmonary MDA levels were assessed at a later time point.

In our study, acute ozone exposure increased *Bnip3* mRNA expression in all offspring except the females from HFD dams, and increased BNIP3 protein expression in all male offspring. Although many studies have examined the effects of ozone exposure on mitochondrial function and content in general, the current study is the first report, to our knowledge, on the ozone effects on specific mitophagy-related markers. The accumulation of mitophagy receptors such as BNIP3 on the mitochondrial outer membrane initiate mitophagy by promoting the binding of the mitochondrial membrane to the autophagosomal membrane [37]. In light of this, the increased abundance of *Bnip3* in our study may have led to a rise in mitophagy as well. Mitophagy aids in maintaining mitochondrial quality [38] and plays an important role in protecting tissues against many forms of injury, such as alcoholic liver disease [39], ischemia/reperfusion-induced acute kidney injury [40], and tumorigenesis [41]. The upregulation of the molecular control of mitophagy in our study may thus be interpreted as a beneficial response, potentially protecting the lung cells against ozone-induced injury. Female offspring from HFD dams were the only offspring to not upregulate *Bnip3* in response to ozone, thereby potentially lacking the protection that an upregulation of mitophagy would offer. This lack of protection against ozone-induced oxidative stress is indeed implied by the increased oxidative status in female offspring from HFD dams, as indicated by the elevated GSSG/GSH ratio and *Sod2* expression. These results suggest that female offspring from dams fed an HFD experienced more detrimental effects from ozone exposure than the other offspring groups. This is in accordance with our previous study [1], which found ozone-induced changes in the gut microbiome metabolites and metabolites indicative of mitochondrial stress in tissues to be more profound in these same female offspring from HFD dams than other offspring groups. For males, the ozone effects did not differ between the offspring from CD or HFD dams. Altogether, these findings suggest that a perinatal HFD influences ozone-induced responses in females, but not in males. The mechanism underlying this sex-specific effect may involve gonadal hormones, as previously suggested by the findings from an ozone exposure study using gonadectomized animals [42]. In this experiment, acute ozone exposure elicited greater detrimental effects in sham-operated females than in males. Moreover, lowering gonadal hormone levels by removing

the ovaries and testes of females and males, respectively, attenuated ozone-induced effects in females, and aggravated ozone responses in males, indicating the association between sex hormones and ozone susceptibility.

Given the upregulation of the molecular control of mitophagy in our study, we hypothesized that abundance of key constituents and regulators of oxidative phosphorylation, biogenesis, fusion, and fission may also be altered. However, the expression of none of the assessed markers was affected by the perinatal HFD or acute ozone exposure. This finding is in contrast with other studies examining the effect of ozone on mitochondria. In a chronic exposure study in mice, ozone exposure downregulated the pulmonary expression of the gene pathways involved in mitochondrial function and decreased the ATP content; oxidative phosphorylation complex I activity; and oxidative phosphorylation complexes I, III, and V protein levels [35]. In a similar study, chronic ozone exposure in mice increased the pulmonary protein levels of DRP1 and oxidative phosphorylation complexes II and IV [34]. Lastly, in an acute exposure study in mice, ozone exposure increased the abundance of MFN2 and oxidative phosphorylation complexes II and IV [36]. Discrepancies between these studies and our study regarding the effect of ozone on mitochondrial markers may possibly be due to differences in the exposure model (chronic vs. acute) or differences in ozone concentrations (≥ 2.5 ppm in other studies vs. 0.8 ppm in our study). In addition, the previously mentioned studies assessed the outcome parameters 24 h after exposure, whereas we measured them within two hours after exposure, which may not have been enough time for the molecular responses to be detectable.

Although the perinatal HFD influenced the molecular responses to ozone, the perinatal HFD on its own (i.e., without the additional ozone exposure) did not have significant effects on the pulmonary oxidant status or molecular regulation of the mitochondrial content. However, maternal obesity has been demonstrated to have negative effects on oxidative stress and mitochondria in other tissues. Saben et al. found that maternal diet-induced metabolic syndrome increased lipid peroxidation and impaired mitochondrial morphology, dynamics, and metabolism in offspring muscle tissue and oocytes [43]. Similarly, Bruce et al. reported a perinatal HFD increased the expression of genes related to oxidative stress and decreased the activity of all electron transport chain complexes in offspring livers [44]. Lastly, in a study by Larsen et al., a prenatal HFD altered the mitochondrial morphology and impaired mitochondrial dynamism in offspring primary cardiomyocytes, with reductions in both fusion and fission events [45]. It should be noted, however, that none of these studies assessed their outcomes in lung tissue, and we were not able to find any studies that did, which may partly explain the difference in findings between our and previous studies.

In the current study, the offspring animals were exposed to ozone concentrations of 0.8 ppm, which is higher than the ozone levels in controlled human intervention studies. It should be considered, however, that animals were exposed during the day, when rats are physically inactive. Humans, in contrast, either during real-life outdoor exposure or in controlled clinical studies, are generally exposed to ozone during physically active periods. Because of this difference, an ozone exposure of 0.8–1.0 ppm in rats is comparable to an exposure of 0.2–0.4 ppm in humans [46].

The ozone exposure in this study took place when the offspring animals were at an age of forty days, which is comparable to a peri-adolescent age in humans. Around this age of forty days, gonadal hormone concentrations rise in rat, with increases of progesterone and estradiol in females and testosterone in males [47]. As these sex hormones have been associated with ozone susceptibility, the sex differences observed in current study may have been different if the offspring animals would have been exposed and assessed at a different age. Nevertheless, exposing rats at an age of forty days is highly relevant, as children at this peri-adolescent age spend a relatively large part of their time outdoors, resulting in the highest real-life ozone exposure. In addition, children and teens, compared with other populations, are especially vulnerable to the effects of breathing ozone [48].

In contrast to what we hypothesized and found in the literature, many of the molecular markers that we measured in this study were not affected by ozone exposure. As already mentioned in this discussion, this may partly be due to the short amount of time between ozone exposure and tissue collection (two hours), potentially providing an insufficient time for molecular responses to develop. It is, in fact, known that although ozone exposure induces lung injury and inflammation immediately after exposure, the peak in these responses is observed the next day [49,50]. It may thus be better for future studies to assess molecular outcomes the day after exposure. Another limitation of our study is that mitochondrial outcomes were assessed only on the level of mRNA and protein expression. Although these outcomes provide valuable insights into the molecular responses to perinatal HFD and ozone exposure, it remains unclear whether they represent changes in mitochondrial function as well. Future studies may address this limitation by additionally assessing functional mitochondrial outcomes, such as the mitochondrial copy number, oxidative phosphorylation enzyme activity, and live imaging of fusion and fission events. In addition, future studies may also expand their focus to other relevant organs, such as the liver, given the involvement of oxidative stress and mitochondria in various liver conditions [51,52], and the adverse effects both perinatal HFD and ozone exposure are known to have on the liver [40,49,50]. Next to this, future studies may use a chronic exposure model instead of the acute exposure used in current study. Combining a perinatal HFD with such chronic ozone exposure may very well lead to different results than those reported in our

study, as previous research has shown that repeated ozone exposure over several days leads to the attenuation of pulmonary responses [12,51,52].

In summary, we showed that acute ozone exposure increased pulmonary oxidant status and upregulated the molecular control of mitophagy, and that in female offspring these responses were exacerbated by a perinatal HFD. Considering the current obesity pandemic and a substantial part of the urban population being exposed to harmful ozone levels, investments should be made into improving early life nutrition and reducing exposure to air pollution to protect society against adverse health effects.

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Supplement

Table S1. Primer sequences for real-time quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<u>Reference genes</u>		
<i>Tubab1b</i>	AGCGCAGCATCCAGTTTGT	CTGTGGTGTTGCTCAGCATAGA
<i>Rpl13a</i>	GCGGATGAACACCAACCCGT	CAGCCTGGCCTCTTTTGGTCT
<i>Ppia</i>	TCCATGGCAAATGCTGGACCAA	CCTGGACCCAAAACGCTCCA
<u>Antioxidants</u>		
<i>Cat</i>	GAATGGCTATGGCTCACACA	CAAGTTTTTGATGCCCTGGT
<i>Sod1</i>	CGAGCATGGGTTCCATGTC	CTGGACCGCCATGTTTCTTAG
<i>Sod2</i>	ATTAACGCGCAGATCATGCA	CCTCGGTGACGTTTCAATTGT
<u>Mitophagy</u>		
<i>Pink1</i>	CCAAACACCTTGGCCTTCTA	CTTAAGATGGCTTCGCTGGA
<i>Bnip3</i>	CAGAGCGGCGAGGAGAACCTGCAG	GCTGCTCCCATTCCCATTGCTGAAG
<i>Bnip3l</i>	AGGCTAACCTGCAGCACAGT	CCTGCGGATGAAACTGCTA
<u>Mitochondrial biogenesis</u>		
<i>Ppargc1a</i>	GGGACATGTGCAGCCAAGACT	GATCTGGGCAAAGAGGCTGGT
<i>Ppargc1b</i>	AGCGCTTCGAGGTGTTTGGT	TTCTCAGGGTAGCGCCGTTT
<i>Tfam</i>	AATTGCAGCCATGTGGAGGGAG	GCCGGGCTTCCTTCTCTAAGC
<u>Mitochondrial fusion</u>		
<i>Mfn1</i>	CGGAGGCATATGAAAGTGGC	CCATCAGTTCCTCCACACT
<i>Mfn2</i>	TTGACTCCAGCCATGTCCAT	GGTGACGATGGAGTTGCATC
<i>Opa1</i>	CAGCTGGCAGAAGATCTCAAG	CATGAGCAGGATTTTGACACC
<u>Mitochondrial fission</u>		
<i>Drp1</i>	TGGAGATGGTGGTCAGGAAC	TTTCGTGCAACTGGAAGTGG
<i>Fis1</i>	ATGGATGCCAGAGATGAAG	ACGATGCCTCTACGGATGTC
<u>Oxidative phosphorylation</u>		
<i>Ndufb3</i>	GAAGAAGCTTGCTGCACGAGG	ACGCAGCAAACCCCATTTG
<i>Sdhb</i>	GAACGGAGACAAGTACCTGGGG	GATGGTGTGGCAGCGGTAGA
<i>Cyc1</i>	ATGTTGCCACCTTCCTTCGCT	AGGACTGACCACTTATGCCGC
<i>Cox4i1</i>	CTGAAGGAGAAGGAGAAGG	CAGTGAAGCCGATGAAGA

Table S2. Immunoprecipitation buffer composition.

Component	Concentration
Tris	50 mM
NaCl	150 mM
Glycerol	10% (w/v)
NP-40	0.05% (w/v)
Ethylenediaminetetraacetic acid (EDTA)	1 mM
cOmplete protease inhibitor cocktail*	1x
PhosSTOP phosphatase inhibitor*	1x
pH	7.4

*Roche, Basel, Switzerland

Table S3. Laemmli buffer (4x).

Component	Concentration
Tris	0.25 M
Sodium dodecyl sulfate (SDS)	8% (w/v)
Glycerol	40% (w/v)
Dithiothreitol (DTT)	0.4 M
Bromophenol Blue	0.02% (w/v)
pH	6.8

Table S4. Western blot primary antibodies.

Antigen	Dilution	Supplier
BNIP3	1:1000	Cell Signaling Technology
BNIP3L	1:1000	Cell Signaling Technology
SOD2	1:1000	Santa Cruz Biotechnology
OXPBOS I-V	1:1000	Abcam

Table S5. Western blot secondary antibodies.

Primary antibody	Secondary antibody	Dilution	Supplier
BNIP3	Anti-rabbit	1:10,000	Vector Laboratories
BNIP3L	Anti-rabbit	1:10,000	Vector Laboratories
SOD2	Anti-mouse	1:10,000	Vector Laboratories
OXPBOS I-V	Anti-mouse	1:10,000	Vector Laboratories

Chapter 4

Perinatal fish oil diet reduces *ex vivo* cytokine levels of polarizing CD4⁺ T cells and induces anemia with microvesicular steatosis in mouse offspring

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In preparation for submission

Abstract

Maternal supplementation with n-3 polyunsaturated fatty acids (PUFAs) during pregnancy is associated with reduced risk of developing asthma in the offspring. However, the mechanisms behind this association are unclear, including the role of progeny CD4⁺ T cells, which are essential to asthma pathology. Therefore, this study examined the effect of a perinatal n-3 PUFA-rich fish oil diet (FOD) on *ex vivo* polarization of CD4⁺ T cells in mouse offspring. For this purpose, dams received either an FOD or a control diet (CD) during gestation and lactation. At the end of lactation, offspring splenic naïve CD4⁺ T cells were isolated and polarized *ex vivo* towards Th1 or Th2 lineage. During polarization, CD4⁺ T cells were examined for secretion and gene expression of Th-specific cytokines and transcription factors. The perinatal FOD significantly reduced extracellular levels of IL-4, IL-5, IL-13, IFN- γ and IL-10 during Th1 polarization, and IL-5, IL-13 and IL-10 during Th2 polarization. The perinatal FOD affected mRNA expression of Th-specific cytokines and transcription factors as well, but these changes were less consistent than the effects observed for extracellular cytokine levels. In addition, the perinatal FOD unexpectedly induced anemia in both dams and offspring, and microvesicular steatosis in offspring liver. These findings suggest that perinatal exposure to high levels of n-3 PUFAs may have both beneficial and adverse effects in the offspring and thus that dosages should be carefully considered for future studies.

Introduction

Allergic asthma is a chronic inflammatory airway disorder, which is characterized by airway hyperreactivity and reversible airway obstruction. Asthma symptoms include shortness of breath, cough, wheezing, chest tightness, and a decreased expiratory airflow. Despite a growing knowledge of asthma, the prevalence of asthma has increased over the past few decades leading to current prevalences of 7.8% in the United States, 9.4% in the United Kingdom, and 5.4% in the European Union [1-4]. Concurrently, consumption of n-3 polyunsaturated fatty acids (PUFAs) has decreased, whereas consumption of n-6 PUFAs has increased, resulting in the previously formulated hypothesis by Black and Sharpe that the increasing prevalence of asthma and other allergic diseases may be explained by changes in the n-6:n-3 ratio of the diet [5]. More recently, epidemiologic studies have indeed found associations between low maternal intake of n-3 PUFAs and increased risk of asthma and asthma-related symptoms in offspring [6-8]. Moreover, intervention studies have shown that maternal supplementation with n-3 PUFAs and n-3 PUFA-rich fish oils during pregnancy may reduce the risk of offspring being diagnosed with asthma, having asthma medication prescribed, or experiencing asthma symptoms [9-11].

For the development of asthma and other allergies, sensitization to allergens is crucial. Sensitization to allergens is thought to occur early in life and involves antigen-induced polarization of CD4⁺ T cells [12,13]. Upon inhalation, the allergen is processed by a dendritic cell, which then displays allergen-derived antigens on its surface, becoming an antigen-presenting cell (APC), and presents the antigen to naïve CD4⁺ T cells in the spleen or lymph nodes. In allergic individuals, naïve CD4⁺ T cells will predominantly polarize into T helper (Th) 2 cells, subsequently leading to hallmark features of asthma, such as B cell isotype switching to IgE, mast cell differentiation and maturation, and eosinophil maturation and survival [13]. However, naïve CD4⁺ T cells may also polarize into other Th subsets, such as Th1, Th17 and regulatory T (Treg) cells. Each of these Th lineages has its own polarization pathway, involving expression of Th-specific transcription factors and cytokines. For Th1 cells, transcription factor T-bet induces expression of cytokine IFN- γ , whereas for Th2 cells, transcription factor Gata3 promotes expression of cytokines IL-4, IL-5, and IL-13. For Th17 cells, secretion of cytokine IL-17 is induced by transcription factor ROR γ t, and for Treg cells, transcription factor Foxp3 induces expression of cytokine IL-10 [14]. Each of these Th subsets and their signature transcription factors and cytokines have their own associations with asthma. For instance, compared with healthy individuals, asthma patients display increased pulmonary levels of Th2 cytokines IL-4 and IL-5 and Th17 cytokine IL-17, and decreased levels of Th1 cytokine IFN- γ and Treg cytokine IL-10 [15-18].

Although CD4⁺ T cells are known to be important for asthma pathology, and despite maternal consumption of n-3 PUFAs being associated with asthma risk in offspring, the effects of maternal n-3 PUFAs on CD4⁺ T cells in offspring have not been studied. We therefore performed a study in which we investigated the effect of a perinatal n-3 PUFA-rich fish oil diet (FOD) on *ex vivo* polarization of CD4⁺ T cells in mouse offspring. In this study, dams received either an FOD or a control diet (CD) during gestation and lactation. At the end of lactation, offspring splenic naïve CD4⁺ T cells were isolated and polarized *ex vivo* towards Th1 or Th2 lineage. During polarization, CD4⁺ T cells were examined for secretion and gene expression of Th-specific cytokines and transcription factors to assess the effect of a perinatal FOD on *ex vivo* polarization of CD4⁺ T cells. In this paper, we show that a perinatal FOD reduced extracellular cytokine levels of both Th1- and Th2-polarizing CD4⁺ T cells isolated from offspring. Furthermore, unanticipated secondary effects were observed, with a perinatal FOD inducing not only anemia in both dams and offspring, but also microvesicular steatosis in offspring liver.

Methods

Animal experiment

Animals were housed in individually ventilated cages, at a temperature of 21°C, relative air humidity of 55±10%, 12/12-hour light/dark cycle, and with *ad libitum* access to feed and water. Specific pathogen-free BALB/cByJ females (Charles River, Écully, France) were densely co-housed in a female-only room for two weeks and subsequently exposed to male pheromones through bedding material to synchronize the females' estrus cycle (Lee-Boot and Whitten effect [19,20]). Females (eight weeks old) were then mated for a maximum of two consecutive nights and included in the experiment if they had a vaginal plug the morning after mating. Plug-positive females were randomly assigned to either a control diet (CD; Research Diets, New Brunswick, NJ, USA) or an FOD (Research Diets). Both diets were identical in composition, except for the fat source, which was soybean oil for the CD and menhaden oil combined with flaxseed oil for the FOD. Diets were designed to have minimal differences in total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), and total PUFA, but opposing amounts of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), total n-3 PUFAs and total n-6 PUFAs (Table 1). Detailed formulas for both diets are listed in Table S1. Diets started on gestational day 0 (GD; number of days after confirmation of vaginal plug) and continued throughout gestation and lactation. To prevent oxidation of the diet, the antioxidant tert-butylhydroquinone was added. Furthermore, the diets were stored in vacuum bags at -20°C and the diets in animal cages were replaced with fresh feed three times per week. Bodyweight was assessed on GD 0, 7, and 14 for dams, and

Table 1. Macronutrient and fatty acid composition of control diet (CD) and fish oil diet (FOD).

	CD	FOD
Macronutrients	(kcal %)	(kcal %)
Fat	12	12
Protein	26	26
Carbohydrate	62	62
Fatty acids	(g/kg)	(g/kg)
EPA	0.00	1.92
DHA	0.00	1.39
n-3 PUFA	3.70	24.43
n-6 PUFA	25.80	6.45
Total SFA	7.54	6.11
Total MUFA	11.66	9.44
Total PUFA	29.53	31.54

In vivo experiment **Figure 1.** Experimental design of animal model. Dams received a control diet or fish oil diet during gestation and lactation. Body weight (BW) was measured on gestational day (GD) 0, 7 and 14 for dams, and on postnatal day (PND) 7, 14 and 21 for both dams and offspring. At the end of lactation (PND 21), both dams and offspring were euthanized and tissues were collected for further analyses. GD 0 and PND 0 are the first day of gestation and lactation, respectively.

on postnatal day (PND; number of days after birth of litter) 7, 14, and 21 for both dams and offspring. At the end of lactation, on PND 21, both dams and offspring were euthanized for tissue collection. Litters were not culled and both male and female offspring were used for assessment of all outcomes. Animal experiments were approved by the Animal Experiment

Committee of Maastricht University (project license number AVD1070020198784). For a schematic overview of the animal model, see Figure 1.

Animal tissue collection

On PND 21, dams received a single subcutaneous dose of 0.05 mg/kg buprenorphine. Thirty minutes later, anesthesia was induced with 4-5% isoflurane and maintained with 1.5-2.5% isoflurane. After confirmation of full anesthesia, dams were exsanguinated via the vena cava and blood was collected in K₂EDTA tubes (BD Biosciences, Franklin Lakes, NJ, USA). Whole blood for hematological assessment was stored on ice and measured within a few hours. To obtain plasma, blood was centrifuged at 1,300 g for 15 min at 4°C and plasma was stored at -80°C until fatty acid and ferritin analyses. Liver and spleen were stored in formalin for histology. Offspring was euthanized using cervical dislocation. Offspring spleen was collected in ice-cold PEB buffer (PBS, pH 7.2, 2 mM EDTA, 0.5% (w/v) bovine serum albumin (BSA); prepared from MACS Rinsing Solution and MACS BSA Stock Solution; Miltenyi Biotec, Bergisch Gladbach, Germany) and directly used for enrichment of naïve CD4⁺ T cells. Offspring liver was snap-frozen in liquid nitrogen and stored at -80°C for gene expression analysis, or stored in formalin for histology.

Enrichment of naïve CD4⁺ T cells

Preparations of single-cell suspensions and enrichment of naïve CD4⁺ T cells were performed sterile and on ice. Freshly harvested spleens were transferred to 1 mL of fresh, ice-cold PEB buffer in a 24-well plate. Spleens were gently smashed with the thumb side of a syringe plunger and filtered through a 30 µm cell strainer (Miltenyi Biotec). Naïve CD4⁺ T helper cells (CD45⁺CD3⁺CD4⁺CD62L^{high}CD44^{low}) were enriched from single-cell suspensions using negative depletion with Naïve CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer's protocol and directly used for *ex vivo* polarization assay. Purity of naïve cells was determined to be ≥90% using flow cytometry (Figure S1). Less than 4% of enriched cells were effector T cells and 0% were memory cells. For flow cytometry, cells were stained with CD45-VioGreen, CD4-VioBlue, CD3-APC, CD62-PE, and CD44-VioBright FITC (all Miltenyi Biotec; catalog numbers listed in Table S2) according to manufacturer's protocol. Samples were measured with BD FACS Canto II and data were analyzed with BD FACSDiva Software v6.1.2 (BD Biosciences).

***Ex vivo* polarization assay**

Naïve CD4⁺ T cells were seeded in 96 well-plates at 5*10⁴ cells/well in TexMACS medium (Miltenyi Biotec), supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 0.01

mM 2-mercaptoethanol, 1% (v/v) Pen Strep (Life Technologies; final concentrations 100 U/ml penicillin and 100 µg/ml streptomycin), Dynabeads Mouse T-activator CD3/CD28 (ThermoFisher Scientific, Waltham, MA, USA) at a 1:1 cell:bead ratio, 10 U/ml IL-2, and polarization-specific cytokines (i.e. 5 ng/ml IL-12 for Th1 polarization or 5 ng/ml IL-4 for Th2 polarization). IL-2, IL-12 and IL-4 were purchased at Miltenyi Biotec (catalog numbers listed in Table S3). Cells were incubated in a humidified CO₂ (5%) incubator at 37°C and harvested at 0, 4, 5, and 6 days after seeding. Cells were centrifuged at 300 g for 5 min, supernatant was stored at -20°C for extracellular cytokine detection, and cell pellets were stored at -80°C for RNA isolation. For a schematic overview of the polarization assay, see Figure 2.

Figure 2. Experimental design of *ex vivo* polarization assay. Directly after euthanasia on postnatal day 21, naïve CD4⁺ T cells were enriched from offspring spleen. T cells were activated with anti-CD3 and anti-CD28, and stimulated to polarize towards Th1 or Th2 lineage with IL-2 and IL-12 or with IL-2 and IL-4, respectively. Cells were cultured for six days and harvested on day 0, 4, 5 and 6 of polarization. In supernatant, Th-specific cytokines were measured. In cells, gene expression of Th-specific transcription factors and cytokines were measured.

Extracellular cytokine concentrations and gene expression of CD4⁺ T cells

Extracellular cytokine levels of *ex vivo* polarizing CD4⁺ T cells were determined using a custom ProcartaPlex 6-plex assay (Thermo Fisher Scientific) according to manufacturer's protocol on a Luminex 100 system (Luminex Corporation, Austin, TX, USA). To assess gene

expression of Th-specific transcription factors and cytokines, RNA was isolated from cell pellets using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's protocol. cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. Real-time quantitative PCR (RT-qPCR) was performed using SensiMix SYBR & Fluorescein Kit (Meridian Bioscience, Cincinnati, OH, USA) with 4.4 µl of 1:10 diluted cDNA and 150 nM of each target-specific primer (Table S4) in a total reaction volume of 10 µl on a LightCycler 480 (Roche, Basel, Switzerland). Gene expression was quantified using LinRegPCR software version 2014.0 [21] and normalized using the geometric mean of expression levels of reference genes *Cd3e* and *Actb*.

Plasma fatty acids, plasma ferritin, hematology, and dietary iron

Concentrations of 58 different free phospholipid fatty acids were measured by gas chromatography – triple quadrupole mass spectrometry in dam plasma according to Schött et al. [22]. Ferritin levels were assessed in dam plasma with Mouse Ferritin ELISA Kit (Crystal Chem, Elk Grove Village, IL, USA) according to manufacturer's protocol. Erythrocyte characteristics were determined in dam whole blood on a Sysmex XN-9000 automated flow cytometer (Sysmex, Kobe, Hyogo, Japan) at Maastricht University Medical Centre Central Diagnostic Laboratory. Dietary iron levels were determined by inductively coupled plasma optical emission spectrometry at NutriControl (Veghel, The Netherlands).

Histology

Formalin-preserved tissues were embedded in paraffin, cut into 4 µm sections and stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) or periodic acid-Schiff-diastase (PAS-D). Snap-frozen tissues were cut into 7 µm sections and stained with Oil Red O (ORO).

Gene expression in offspring liver

For offspring liver gene expression, 50 – 100 mg snap-frozen tissue was homogenized in TRI-Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and RNA was isolated using standard phenol/chloroform extraction. 300 ng RNA was converted into cDNA with the SensiFAST cDNA Synthesis Kit according to manufacturer's protocol. For RT-qPCR, SensiMix SYBR & Fluorescein Kit (Meridian Bioscience) was used with 5 µl of 7.5x diluted cDNA and 300 nM target-specific primers (Table S4) in a total reaction volume of 15 µl. Expression levels were quantified using LinRegPCR software version 2014.0 [21] and normalized with the geometric mean of *Gapdh* and *Hprt* expression levels.

Statistics

All researchers were blinded for diet allocation up until data analysis. A priori power calculations determined that optimal group sizes would be 33 pups per group for two-sided $\alpha = 0.05$ and power of 0.80. To obtain this amount of pups, 36 dams per group started breeding procedures. In the end, seven vs. six dams (CD vs. FOD, respectively) gave birth to a viable litter, resulting in 35 vs. 27 pups (CD vs. FOD) that finished the experiment.

Based on density and Q-Q plots (with Q-Q lines), data distributions were improved by $\log_{10}$ -transformation for CD4⁺ T cell extracellular cytokine levels; by square root-transformation for CD4⁺ T cell gene expression; and by $\log_2$ -transformation for offspring liver gene expression. Population characteristics were analyzed with unpaired, two-tailed t-test (for litter size at birth), Poisson regression with diet as factor (for gestational age at birth), or Fisher's exact test (for offspring sex and offspring mortality). Overall fatty acid profiles were visualized with principle component analysis using standardized fatty acid concentrations. The effect of FOD on individual and grouped fatty acid levels and on plasma ferritin levels was analyzed with unpaired, two-tailed t-tests. For offspring and dam body weight, offspring liver gene expression, and cytokine levels and gene expression assessed in the polarization assays, data were analyzed with the linear mixed-effects models. For these mixed-effects models, time and a diet*time interaction were used as fixed factors when assessing dam outcomes, whereas for offspring outcomes, sex, time and a diet*time interaction were used as fixed factors.

Results were graphically presented as boxplots, with boxes representing first quartile, median and third quartile, and whiskers representing minimum and maximum values within 1.5 times the interquartile range (IQR) below the first quartile or above the third quartile, respectively, and with points representing values outside the limits of the whiskers. $P < 0.05$ was considered significant. All statistical analyses were performed in R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) with nlme package version 3.1-152 for linear mixed-effects model.

Results

Perinatal FOD alters fatty acid profile in dams

To study the effect of maternal n-3 PUFAs on *ex vivo* polarization of offspring CD4⁺ T cells, dams were fed an FOD or CD during gestation and lactation. First, to assess whether the perinatal FOD indeed changed maternal fatty acid status, 58 different fatty acids were measured in maternal plasma at the end of lactation. Principle component analysis of these

Figure 3. Perinatal fish oil diet (FOD) alters maternal plasma fatty acid profile. A) Principle component analysis of 58 fatty acids. B) Eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) levels. C) Total n-3 fatty acids, total n-6 fatty acids, total polyunsaturated fatty acids (PUFA), total monounsaturated fatty acids (MUFA), and total saturated fatty acids (SFA) levels. Fatty acids were measured in dam plasma at the end of lactation (postnatal day 21), after dams received a control diet (CD) or FOD during gestation and lactation. Data are presented as A) scatter plot of the first and second principle component (PC), covering 28% and 15% of the total variance, respectively, with each dot representing one dam; or as boxplots (B and C). Statistical analysis by unpaired, two-tailed t-test (B and C). n = 6 per group. **p<0.01, ***p<0.001.

maternal plasma fatty acid data showed that FOD dams had a different overall fatty acid status than CD dams (Figure 3a). Furthermore, in accordance with the diet, FOD dams had significantly increased levels of EPA, DHA and total n-3 PUFAs, and significantly decreased levels of total n-6 PUFAs, compared with CD dams (Figure 3b-c). Although the FOD was designed to have similar levels of total PUFA, total MUFA and total SFA when compared with the CD, plasma levels of total PUFA and total SFA were significantly lower in FOD dams compared with CD dams (Figure 3c). Total MUFA levels were not different between both groups. Individual concentrations of all 58 measured fatty acids are listed in Table S5. Altogether, these findings indicate that a perinatal FOD significantly altered the maternal fatty acid profile.

Perinatal FOD decreases offspring bodyweight during lactation

To check whether the perinatal FOD affected the animals' general health, several baseline characteristics were assessed. As expected, no significant differences were observed between CD and FOD animals for gestational duration, litter size, offspring sex and offspring mortality (Table 2). In addition to these characteristics, dam bodyweight was measured during gestation and lactation, and offspring bodyweight was measured during lactation. Both CD and FOD dams gained similar amounts of bodyweight during gestation and remained constant in bodyweight during lactation (Figure 4a-b). Offspring from both CD and FOD dams increased bodyweight during lactation, but for offspring from FOD dams, weight gain was significantly lower than for offspring from CD dams (Figure 4c). In summary, these findings show that a perinatal FOD decreased offspring bodyweight during lactation.

Table 2. Population characteristics. No significant differences were found between control diet (CD) and fish oil diet (FOD) for any of the parameters. Statistical analysis by Poisson regression (gestational age at birth), unpaired, two-tailed t-test (litter size at birth), or Fisher's exact test (offspring sex and offspring deceased during lactation). All numbers represent count data, except litter size at birth, which represents mean and 95% confidence interval (CI).

	CD	FOD
Dams with viable litter	7	6
Gestational age at birth		
19 days	3	1
20 days	4	4
21 days	0	1
Litter size at birth (mean (95% CI))	5.3 (3.4 – 7.2)	4.8 (3.0 – 6.6)
Offspring born	37	29
Offspring sex (female/male)	25/12	15/14
Offspring deceased during lactation	2	2
Offspring included <i>in ex vivo</i> experiment	35	27

Figure 4. Perinatal fish oil diet (FOD) decreases offspring bodyweight during lactation. A) Dam bodyweight during gestation, $n = 7$ vs. 6 (control diet (CD) vs. FOD). B) Dam bodyweight during lactation, $n = 7$ vs. 6. C) Offspring bodyweight during lactation. $n = 35$ vs. 27, $***p < 0.001$ for corresponding diet*day interaction. Dams received CD or FOD during gestation and lactation. Data are presented as boxplots. Statistical analysis by linear mixed-effect models.

Perinatal FOD induces anemia in both dams and offspring

To examine whether the decreased bodyweight in offspring from FOD dams was related to an underlying pathology, we performed a number of additional secondary analyses in a subgroup of dams and offspring. Since offspring from FOD dams appeared to be lethargic, tissues were first examined for signs of anemia. Indeed, spleens from FOD dams, as well as livers from offspring from FOD dams displayed extramedullary hematopoiesis, which is a clear indication of anemia, whereas no extramedullary hematopoiesis was observed in spleens from CD dams and livers from offspring from CD dams (Figure 5a-d). Anemia diagnosis was further supported by hematological assessment of two FOD dams and one CD dam, which showed that both FOD dams had lower red blood cell count, hemoglobin concentration and mean corpuscular hemoglobin, and higher relative distribution width, compared with CD (Table S6). In addition, both FOD dams had lower mean corpuscular volume than CD, suggesting that the FOD-induced anemia may be related to iron deficiency rather than a vitamin deficiency [23]. This suggestion was further examined by measuring different iron-related markers. First, iron levels were measured in both diets, but these did not differ between the FOD and CD (47 mg/kg diet for both CD and FOD). Moreover, iron levels in CD and FOD were above the levels advised for laboratory animals by the NRC (35 mg/kg diet) [24]. In addition, serum ferritin concentration, which is a marker for total iron storage in the body, was measured in dam plasma. Although not statistically significant, ferritin levels tended to be lower in FOD dams than in CD dams (Figure 6a). Lastly,

Figure 5. Perinatal fish oil diet (FOD) induces extramedullary hematopoiesis in dams and offspring, and hepatic microvesicular steatosis in offspring. A) Hematoxylin-eosin (HE)-stained spleen from control diet (CD)-fed dams. B) HE-stained spleen from FOD-fed dams showing extramedullary hematopoiesis (dark purple cells). C) HE-stained liver from offspring from CD-fed dams. D) HE-stained liver from offspring from FOD-fed dams showing extramedullary hematopoiesis (dark cells at arrows) and microvesicular steatosis (sponge-like cells). E) Oil Red O (ORO)-stained liver from offspring from CD-fed dams. F) ORO-stained liver from offspring from FOD-fed dams showing increased lipid accumulation. Tissues were collected at the end of lactation (postnatal day 21) after dams received a CD or FOD during gestation and lactation. Images were taken at 200x magnification. Representative images are shown, n = 3 per group.

transcription levels of the hepcidin gene (*Hamp*), which is a master regulator of systemic iron metabolism [25], were measured in offspring liver, but no differences were found between offspring from FOD dams and offspring from CD dams (Figure 6b). Altogether, these data suggest that the perinatal FOD induced anemia in both dams and offspring.

Perinatal FOD induces microvesicular steatosis in offspring liver

During the examination of offspring liver for histological signs of anemia, an additional, unanticipated observation was made: livers of offspring from FOD dams displayed microvesicular steatosis, which was not present in livers of offspring from CD dams (Figure 5c-d). Additional lipid staining of these tissues showed enhanced lipid accumulation in livers

Figure 6. Effect of perinatal fish oil diet (FOD) on iron-related markers. A) Dam plasma ferritin levels, $n = 5$ vs. 6 (control diet (CD) vs. FOD), statistical analysis by unpaired, two-tailed t-test. B) Gene expression of Hepcidin gene *Hamp* in offspring liver, $n = 31$ vs. 24, statistical analysis by linear mixed-effect models. Both maternal plasma and offspring liver were collected at the end of lactation (on postnatal day 21) after dams received a CD or FOD during gestation and lactation. Data are presented as boxplots.

of offspring from FOD dams, whereas livers of offspring from CD dams only showed mild lipid accumulation (Figure 5e-f). No signs of inflammation, apoptosis or necrosis were found in livers of offspring from both CD and FOD dams. Furthermore, PAS staining of offspring livers showed no differences in hepatic glycogen levels between offspring from CD and FOD dams (results not shown). Histological examination of offspring heart, lung, thymus, kidney, pancreas, salivary gland, and gastrointestinal tract did not show any abnormalities (results not shown). In summary, these results indicate that a perinatal FOD induced microvesicular steatosis in offspring liver.

Perinatal FOD reduces extracellular cytokine levels of ex vivo polarizing CD4⁺ T cells

To examine the effect of a perinatal FOD on CD4⁺ T cell polarization, we set up an assay in which naïve CD4⁺ T cells were isolated from offspring spleen at the end of lactation and polarized *ex vivo* towards Th1 or Th2 lineage. In this *ex vivo* polarization assay, Th1 polarization induced elevation of extracellular levels of IL-4, IL-5, IL-13, IFN- γ and IL-10, with the largest elevation for IFN- γ . In addition, Th1 polarization increased mRNA expression of cytokine genes *Il4*, *Il5*, *Il13*, *Ifng* and *Il10*, and of transcription factors *Tbx21* and *Foxp3*. On the other hand, Th1 polarization decreased mRNA expression of transcription factor *Gata3*. Th1 polarization did not affect any Th17 marker (i.e., extracellular levels of IL-17a, and mRNA expression of *Il17a* and *Rorc*). To study the effect of perinatal FOD on CD4⁺ T cell polarization, these polarization responses were compared between cells isolated from CD offspring and cells isolated from FOD offspring. This showed that the perinatal FOD altered

polarization-induced responses, with most profound effects on extracellular cytokine levels. Extracellular cytokine levels were significantly lower in the FOD group than the CD group for IFN- γ , IL-13 and IL-10 on polarization days 4, 5 and 6; for IL-5 on polarization days 4 and 6; and for IL-4 on polarization day 4 (Figure 7a-e). The perinatal FOD also affected polarization-induced changes in gene expression, but these effects were less consistent than the FOD effects on extracellular cytokine levels. mRNA levels of *Il4*, *Il5*, and *Il13* were significantly lower in the FOD group than the CD group on polarization day 4, whereas expression of *Gata3* was significantly higher in the FOD group than the CD group on polarization days 4 and 5, as was expression of *Foxp3* on polarization day 4 (Figure 7f-j). Gene expression of *Ifng*, *Il10* and *Tbx21* were not affected by the perinatal FOD (Figure S2).

Th1 polarization assay

Figure 7 (continued on next page).

Figure 7. Perinatal fish oil diet (FOD) reduces extracellular cytokine levels of *ex vivo* Th1-polarizing CD4⁺ T cells. Extracellular levels of IFN- γ (A), IL-13 (B), IL-10 (C), IL-5 (D) and IL-4 (E), and mRNA expression of *Il4* (F), *Il5* (G), *Il13* (H), *Gata3* (I), and *Foxp3* (J) were measured at 0, 4, 5 and 6 days after incubation of naïve CD4⁺ T cells in presence of α -CD3, α -CD28, IL-2 and IL-12. Splenic naïve CD4⁺ T cells were isolated at the end of lactation from offspring after dams received a control diet (CD) or FOD during gestation and lactation. Data are presented as boxplots. Statistical analysis by linear mixed-effects model. $n = 32$ -35 vs. 26-27 (A – E; CD vs. FOD) or $n = 33$ -34 vs. 25-27 (F – J; CD vs. FOD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for corresponding diet*day interaction.

In the *ex vivo* polarization assay, Th2 polarization increased extracellular levels of IL-5, IL-13, IFN- γ and IL-10, with the smallest elevation for IFN- γ . Furthermore, Th2 polarization increased gene expression of cytokine genes *Il4*, *Il5*, *Il13* and *Ifng*, and of transcription factors *Gata3* and *Tbx21*, but not of *Il10* and *Foxp3*. Th2 polarization did not affect any Th17 marker (i.e., extracellular levels of IL-17a, and mRNA expression of *Il17a* and *Rorc*). Comparing polarization responses between cells from CD offspring and cells from FOD offspring showed that similar to Th1 polarization, the perinatal FOD also affected Th2 polarization-induced responses, again with most consistent effects on extracellular cytokine levels, with extracellular levels of IL-5, IL-13 and IL-10 being significantly lower in the FOD group than in the CD group (Figure 8a-c). IFN- γ levels, however, did not differ between FOD and CD (Figure S3). On the level of gene expression, the perinatal FOD only affected expression of *Il13* and *Gata3*, for which mRNA levels were significantly higher in the FOD group than the CD group on polarization day 6 or on polarization day 4, respectively (Figure 8d-e). Expression levels of *Il4*, *Il5*, *Ifng*, *Il10*, *Tbx21* and *Foxp3* were not affected during Th2 polarization by the perinatal FOD (Figure S3). Altogether, these results show that a perinatal FOD reduced extracellular cytokine levels of *ex vivo* Th1- and Th2-polarizing offspring CD4⁺ T cells.

Figure 8. Perinatal fish oil diet (FOD) reduces extracellular cytokine levels of *ex vivo* Th2-polarizing CD4⁺ T cells. Extracellular levels of IL-5 (A), IL-13 (B) and IL-10 (C), and mRNA expression of *IL13* (D) and *Gata3* (E) were measured at 0, 4, 5 and 6 days after incubation of naïve CD4⁺ T cells in presence of α -CD3, α -CD28, IL-2 and IL-4. Splenic naïve CD4⁺ T cells were isolated at the end of lactation from offspring after dams received a control diet or FOD during gestation and lactation. Data are presented as boxplots. Statistical analysis by linear mixed-effects model. $n = 34$ vs. 27 (A – C; CD vs. FOD) or $n = 23$ - 30 vs. 21 (D – E; CD vs. FOD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for corresponding diet*day interaction.

Discussion

Previous research has shown that maternal supplementation with n-3 PUFAs during pregnancy is associated with reductions of asthmatic features in offspring [9-11]. The underlying mechanisms, however, are unclear. Since a skewed polarization of CD4⁺ T cells is involved in asthmatic development, we studied the effect of a perinatal n-3 PUFA-rich FOD on *ex vivo* polarization of CD4⁺ T cells in mouse offspring. In this study, we showed that a perinatal FOD reduced extracellular cytokine levels of both Th1- and Th2-polarizing CD4⁺ T cells. Furthermore, we found that a perinatal FOD induced not only anemia in both dams and offspring, but also microvesicular steatosis in offspring liver.

As expected, the perinatal FOD altered the maternal plasma fatty acid profile, with higher plasma levels of EPA, DHA and total n-3 PUFAs; lower plasma levels of total n-6 PUFAs; and similar levels of total MUFAs in FOD dams compared with CD dams. On the other hand, total PUFAs and total SFAs were designed to be equal in both diets, but resulted in lower plasma levels in FOD dams compared with CD dams. For total SFAs, this discrepancy between dietary levels and plasma phospholipid levels is most likely a result of metabolism, since SFAs are not only taken up from the diet, but also synthesized *de novo*. PUFAs, in contrast, cannot be synthesized *de novo* by mammals, so total plasma PUFA concentrations were expected to reflect dietary intake levels. However, it is known from literature that for α -linolenic acid, which is the main n-3 PUFA in the CD and FOD, plasma phospholipid levels do not correlate with dietary intake levels [26]. As a result, the difference in total n-3 PUFA plasma levels between FOD and CD dams was smaller than the difference in total n-6 PUFA plasma levels, and as a consequence, total PUFA plasma levels were lower for FOD dams than for CD dams. Altogether, these findings indicate that the perinatal FOD had the intended effect on maternal plasma FA levels.

The perinatal FOD unexpectedly induced anemia in both dams and offspring. Although this observation was unanticipated, studies have previously indicated a potential link between diets high in n-3 PUFAs and anemia. Both Miret et al. and Rodriguez et al. demonstrated that feeding rats an FOD induced oxidative stress in erythrocytes [27,28], which in turn increases erythrocyte aging and removal by macrophages [29,30]. For the studies by Miret et al. and Rodriguez et al., erythrocyte counts were not affected and anemia was not observed, since the increased aging and removal of erythrocytes was compensated by an increased erythropoiesis, as indicated by elevated reticulocyte counts, decreased iron stores in liver and spleen, and increased iron absorption [27,28]. This corresponds with our study, since FOD dams did show extramedullary hematopoiesis, but no external signs of anemia, suggesting that any potential FOD-induced increase in erythrocyte turnover was indeed compensated by enhanced erythropoiesis. However, pups from FOD dams did show

anemic symptoms, such as lethargy and reduced bodyweight. We hypothesize that the exposure to the high n-3 PUFA levels of these pups occurred during such a crucial developmental period, that the suggested increased erythrocyte turnover could not be compensated by accelerated erythropoiesis and hence led to observable anemia.

Next to anemia, the perinatal FOD also induced microvesicular steatosis in livers from FOD offspring. Microvesicular steatosis is the accumulation of small lipid droplets in the cytoplasm, leaving the nucleus in the center of the cell [31]. This is in contrast with macrovesicular steatosis, which is characterized by the cytoplasm being filled with larger lipid vacuoles and displacement of the nucleus to the periphery of the cell. Macrovesicular steatosis is a more common form of lipid accumulation and can be observed, for instance, in cases of obesity, insulin resistance and alcoholism. Microvesicular steatosis, on the other hand, is rare and is associated with intoxications and diseases which involve an impairment of mitochondrial β -oxidation [32]. The observation of hepatic lipid accumulation in offspring from FOD dams in our study was unexpected, since n-3 PUFAs are known to reduce hepatic lipid content through the reduction of lipogenesis and elevation of fatty acid oxidation and VLDL secretion [33]. Possibly, it is due to the perinatal period during which pups were exposed to the FOD which led to the observed hepatic lipid accumulation. During this crucial developmental period, pups may have been more vulnerable to the hepatic effects of the perinatal FOD than the adult animals that are usually used in experiments. Nevertheless, presence of the characteristic microvesicular structure in our study, in contrast to the macrovesicular structure, may be explained by the high n-3 PUFA content of the FOD. Previous studies have shown that in diabetic patients, increased fat droplet size in fatty livers was associated with reduced hepatic content of EPA and vice versa [34]. Similarly, a mouse model for hepatic microvesicular steatosis had an identical hepatic n-6:n-3 PUFA ratio as lean mice, whereas mice with hepatic macrovesicular steatosis had a higher hepatic n-6:n-3 PUFA ratio [35]. Moreover, n-3 PUFA supplementation of *ob/ob* mice has previously been shown to convert macrovesicular steatosis into microvesicular steatosis [36].

Using an *ex vivo* polarization assay, we showed that a perinatal FOD affected *ex vivo* Th1 and Th2 polarization of naïve CD4⁺ T cells. Most prominently, the perinatal FOD significantly reduced extracellular levels of IL-4, IL-5, IL-13, IFN- γ and IL-10 during Th1 polarization, and IL-5, IL-13 and IL-10 during Th2 polarization. The perinatal FOD affected mRNA expression of Th-specific cytokines and transcription factors as well, but these changes were less consistent than the effects observed for extracellular cytokine levels. Although mRNA and protein levels do not necessarily have to correlate, as previously indicated [37], the discrepancy between FOD-induced effects on gene and protein expression may suggest that the decreased extracellular cytokine levels observed for FOD offspring were a result of a reduced number of cytokine-secreting cells, rather than a reduced cytokine secretion per

cell. Indeed, previous studies have shown that feeding mice an n-3 PUFA-rich diet reduces the *ex vivo* proliferative capacity of CD4⁺ T cells [38-42]. Next to this, *ex vivo* proliferation of CD4⁺ T cells can be reduced by hypoxia as well [43,44], suggesting that the observed decrease in extracellular cytokines levels during *ex vivo* polarization of CD4⁺ T cells may have been a consequence of the perinatal FOD-induced anemia, rather than being a direct effect of the perinatal FOD.

For all experimental outcomes measured in offspring animals, statistical analyses were adjusted for offspring sex. This approach was determined a priori, since sex hormones are known to influence immune responses [45]. In the current study, however, none of the performed analyses showed a significant effect of sex. This lack of sex effect may be due to the relatively young age at which outcomes were assessed in offspring animals. At this pre-pubertal age, sex hormone levels are still relatively similar for male and female mice [46]. Thus, hormonal differences between male and female offspring in the current study may not have been sufficiently developed to induce sex differences.

A limitation of our study is that the hypothesized decrease in CD4⁺ T cell proliferation could not be confirmed, since cell proliferation was not assessed. As a consequence, it remains unclear whether the perinatal FOD-induced decrease in cytokine levels was related to a lower cytokine production per cell, to lower cell numbers, or a combination of both. Next to this, outcomes related to the polarization assay were assessed only at the whole-sample level. Although these outcomes provide valuable insights in the influence of a perinatal FOD on *ex vivo* CD4⁺ T cell polarization, it remains unclear what changes occurred at the single-cell level. For instance, Th1-polarizing cells were found to produce not only Th1 cytokine IFN- γ , but also Th2-cytokines IL-4, IL-5 and IL-13, and Treg cytokine IL-10. Additionally measuring these cytokines intracellularly at the single-cell level using flow cytometry, would have shown to what extent these cytokines were produced by distinct cell populations.

A particular strength of this study is the use of linear mixed-effects models for analysis of outcomes assessed in offspring. When studying the effect of a prenatal treatment, randomization can only be applied to the dam, whereas outcomes are measured in individual offspring animals. Since animals from the same litter are more alike compared with animals from different litters, this introduces the so-called litter effects. Ignoring these litter effects by analyzing data with standard methods such as t-test or linear regression may lead to increased false positive rates or low power [47]. Using linear mixed-effects models, in contrast, allows to take into account the multilevel structure of the experiment, while at the same time being able to adjust for covariates such as offspring sex.

For future studies, different approaches to both the animal model and the polarization assay may be applied. For the animal model, BALB/cByJ mice were used, since animals from this strain have been shown to develop a Th2-dominant immune response [48-50]. This mimics the Th2-dominant immune response that characterizes patients with asthma and other atopic conditions. It would, however, be interesting to study the effect of a perinatal FOD in other strains as well, such as the more Th1-dominant C57BL/6 strain. For the polarization assay, CD4⁺ T cells were activated by incubating them *ex vivo* with anti-CD3 and anti-CD28. While this induction with exogenous molecules has been widely applied to study CD4⁺ T cell functions, it differs from the *in vivo* situation, in which naïve CD4⁺ T cells are activated by endogenous APCs. Future studies may therefore mimic the *in vivo* situation more by activating naïve CD4⁺ T cells *ex vivo* with endogenous APCs. Alternatively, future studies may examine the effect of a perinatal FOD on CD4⁺ T cell polarization *in vivo*, for example by inducing an allergic response in offspring animals through sensitization and challenge with an allergen, rather than isolating naïve CD4⁺ T cells and polarizing them *ex vivo*.

The effects observed in our study may be considered as both beneficial and adverse. As a beneficial effect, the perinatal FOD reduced extracellular cytokine levels of Th1- and Th2-polarizing CD4⁺ T cells. This reduced cytokine response upon polarization may, in part, explain the positive effects of prenatal supplementation with fish oil on asthma-related outcomes that were previously reported in clinical studies [9-11]. On the other hand, the perinatal FOD in our study induced anemia and microvesicular hepatic steatosis. These adverse effects may be the result of the relatively high dose of n-3 PUFAs present in the FOD diet, since the daily consumption of n-3 PUFAs per kg of bodyweight was approximately 10 times higher for the dams in our study compared with the pregnant women in the COPSAC study [9]. Thus, our findings stress the need to carefully consider dosages for future studies, both *in vivo* and clinical. The Inuit for instance, which have a high intake of n-3 PUFAs, are known to have a high prevalence of unexplained anemia (i.e. anemia which is not related to a micronutrient deficiency) [51], suggesting that a high intake of n-3 PUFAs could potentially lead to adverse effects in humans as well.

In summary, we showed that a perinatal FOD decreased extracellular cytokine levels of CD4⁺ T cells during *ex vivo* Th1 and Th2 polarization. Furthermore, the perinatal FOD induced anemia in both dams and offspring, and microvesicular steatosis in offspring liver. More research is needed to unravel the molecular mechanisms that underlie these effects and to find out what share they have in the beneficial effects of maternal n-3 PUFA supplementation on offspring asthma risk.

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Supplement

Table S1. Detailed formulas for control diet (CD) and fish oil diet (FOD).

	CD (g/kg)	FOD (g/kg)
Protein		
Casein	261	261
L-cysteine	4	4
Carbohydrate		
Corn starch	354	354
Maltodextrin 10	110	110
Dextrose	150	150
Fiber		
Cellulose, BW200	75	75
Inulin	25	25
Fat		
Soybean oil	54	-
Menhaden oil	-	15
Flaxseed oil	-	40
Cholesterol	0.08 ¹	0.08 ²
Minerals		
Mineral Mix S10026	10	10
Dicalcium Phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate	16.5	16.5
Vitamins		
Vitamin mix V10001	10	10
Choline bitartrate	20	20
Antioxidant		
Tert-butylhydroquinone	2.7 mg/kg	2.7 mg/kg

¹ Added to match FOD; ² Naturally occurring in menhaden oil

Figure S1. Purity of naïve CD4⁺ T cells after enrichment from spleen single-cell suspension. Cells were gated on singlet lymphocytes in FSC/SSC and set as 100%. CD4⁺ T cells were gated on CD45⁺ (Q2+Q4) and CD3⁺/CD4⁺ (Q2-1), and subsequently distinguished between naïve (CD62L^{high}CD44^{low}; Q4-2), effector (CD62L^{low}CD44^{low}; Q3-2), central memory (CD62L^{low}CD44^{high}; Q1-2), and effector memory (CD62L^{high}CD44^{high}; Q2-2).

Table S2. Flow cytometry antibodies.

Antibody	Manufacturer	Catalog number
CD62-PE	Miltenyi Biotec	130-112-836
CD4-VioBlue	Miltenyi Biotec	130-118-696
CD3-APC	Miltenyi Biotec	130-122-943
CD44-VioBright FITC	Miltenyi Biotec	130-120-287
CD45-VioGreen	Miltenyi Biotec	130-110-803

Table S3. Cell culture antibodies and cytokines.

Antibody/cytokine	Manufacturer	Catalog number
Dynabeads Mouse T-activator CD3/CD28	Thermo Fisher Scientific	11452D
IL-2, mouse	Miltenyi Biotec	130-120-330
IL-12, mouse	Miltenyi Biotec	130-096-708
IL-4, mouse	Miltenyi Biotec	130-097-757

Table S4. Real-time quantitative PCR primer sequences (5'-3').

Gene	Forward primer	Reverse primer
Ex vivo polarization		
<i>Ifng</i>	ATGAACGCTACACACTGCATC	CCATCCTTTGCCAGTTCCTC
<i>Tbx21</i>	AGCAAGGACGGCGAATGTT	GGGTGGACATATAAGCGGTTC
<i>Il4</i>	ATCATCGGCATTTTGAACGAGG	TGCAGCTCCATGAGAACTA
<i>Il5</i>	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCTG
<i>Il13</i>	TGAGCAACATCACACAAGACC	GGCCTTGCGTTACAGAGG
<i>Gata3</i>	TCCTTGCTACTCAGGTGATCG	TGACCACACTGCACACTGAT
<i>Il10</i>	AGCCTTATCGGAAATGATCCAGT	GGCCTTGTAGACACCTTGGT
<i>Foxp3</i>	CCCATCCCCAGGAGTCTTG	ACCATGACTAGGGGCACTGTA
<i>Il17a</i>	TTTAACTCCCTTGGCGCAAAA	CTTCCCTCCGCATTGACAC
<i>Rorc</i>	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT
<i>Cd3e</i>	ATGCGGTGGAACATTTCTGG	GCACGTCAACTCTACACTGGT
<i>Actb</i>	ATGGAGGGGAATACAGCCC	TTCTTGCAGCTCCTTCGTT
Offspring liver		
<i>Hamp</i>	GCTGCCTGTCTCCTGCTTCT	GCTCTGTAGTCTGTCTCATCTGTTGAT
<i>Gapdh</i>	CATTGTGGAAGGGCTCATGA	GCCCCACGGCCATCA
<i>Hprt</i>	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG

Table S5. Individual concentrations of free phospholipid fatty acids measured in dam plasma at the end of lactation (on postnatal day 21) after dams received a control diet (CD) or fish oil diet (FOD) during gestation and lactation. Statistical analysis by unpaired, two-tailed t-test, n = 6 per group. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; 95% CI, 95% confidence interval.

	CD (mM) mean (95% CI)	FOD (mM) mean (95% CI)	p-value
SFA			
C14.0	0.049 (0.038-0.059)	0.047 (0.036-0.058)	0.815
C16.0	12.376 (11.846-12.906)	11.501 (10.418-12.584)	0.092
C17.0	0.119 (0.099-0.139)	0.156 (0.133-0.18)	0.011
C18.0	8.474 (8.027-8.92)	6.043 (5.342-6.745)	<0.001
C19.0	0.011 (0.009-0.013)	0.012 (0.009-0.015)	0.38
C20.0	0.038 (0.036-0.04)	0.033 (0.029-0.038)	0.05
C21.0	0.003 (0.002-0.003)	0.002 (0.002-0.003)	0.302
C22.0	0.085 (0.079-0.09)	0.067 (0.062-0.072)	<0.001
C23.0	0.053 (0.05-0.057)	0.044 (0.042-0.046)	<0.001
C24.0	0.061 (0.058-0.065)	0.066 (0.062-0.071)	0.054
C25	0.001 (0.001-0.002)	0.001 (0.001-0.001)	<0.001
MUFA			
C14.1n.5trans	0.021 (0.014-0.028)	0.019 (0.014-0.025)	0.725
C14.1n.5	0.039 (0.03-0.047)	0.043 (0.036-0.05)	0.327
C16.1n.7trans	0.027 (0.024-0.03)	0.037 (0.03-0.045)	0.007
C16.1n.7	0.11 (0.096-0.123)	0.077 (0.057-0.097)	0.006
C16.1n.9	0.236 (0.201-0.27)	0.303 (0.236-0.37)	0.044
C17.1n.7trans	0.021 (0.017-0.025)	0.023 (0.019-0.028)	0.322
C17.1n.7	0.028 (0.024-0.031)	0.027 (0.017-0.037)	0.868
C18.1n.9trans	0.045 (0.039-0.051)	0.036 (0.033-0.04)	0.01
C18.1n.7.trans	0.029 (0.025-0.033)	0.027 (0.021-0.033)	0.566
C18.1n.12trans	0.012 (0.009-0.015)	0.005 (0.003-0.008)	0.001
C18.1n.9	4.895 (4.556-5.235)	4.983 (4.446-5.519)	0.731
C22.1n.9	0.014 (0.005-0.023)	0.006 (0.002-0.011)	0.08
C24.1n.9	0.185 (0.171-0.199)	0.231 (0.207-0.255)	0.002
PUFA			
C18.2n.6.tt	0.016 (0.014-0.018)	0.01 (0.009-0.012)	<0.001
C18.2n.6cc	9.87 (9.382-10.357)	6.27 (5.338-7.201)	<0.001
C18.3n.6	0.056 (0.053-0.06)	0.024 (0.019-0.028)	<0.001

C20.2n.6	0.088 (0.072-0.104)	0.065 (0.06-0.071)	0.006
C20.3n.6	0.71 (0.588-0.832)	0.374 (0.347-0.401)	<0.001
C20.4n.6	5.624 (5.273-5.974)	1.193 (0.896-1.49)	<0.001
C22.2n.6	0.046 (0.042-0.05)	0.067 (0.051-0.083)	0.009
C24.1n.9	0.185 (0.171-0.199)	0.231 (0.207-0.255)	0.002
C22.4n.6	0.057 (0.053-0.061)	0.015 (0.014-0.017)	<0.001
C22.5n.6	0.256 (0.231-0.281)	0.046 (0.04-0.051)	<0.001
C24.4n.6	0.036 (0.028-0.045)	0.048 (0.033-0.062)	0.115
C24.5n.6	0.009 (0.008-0.01)	0.005 (0.004-0.006)	<0.001
C18.3n.3	0.055 (0.048-0.061)	0.318 (0.175-0.462)	0.001
C18.4n.3	0.001 (0.001-0.001)	0.003 (0.002-0.004)	<0.001
C20.3n.3	0.017 (0.015-0.019)	0.04 (0.027-0.053)	0.001
C20.4n.3	0.004 (0.003-0.005)	0.141 (0.082-0.201)	<0.001
C20.5n.3	0.108 (0.086-0.129)	2.651 (1.707-3.594)	<0.001
C22.3n.3	0.029 (0.027-0.031)	0.028 (0.024-0.031)	0.533
C22.5n.3	0.089 (0.082-0.096)	0.325 (0.266-0.385)	<0.001
C22.6n.3	2.682 (2.559-2.805)	3.739 (3.356-4.122)	<0.001
C24.5n.3	0.004 (0.004-0.004)	0.009 (0.008-0.01)	<0.001
C24.6n.3	0.031 (0.024-0.037)	0.051 (0.04-0.061)	0.002

Table S6. Red blood cell characteristics in dams after being fed a control diet (CD) or fish oil diet (FOD) during gestation and lactation. Characteristics were measured in whole blood obtained from dams at the end of lactation (on postnatal day 21). RBC: red blood cell count; Hb: hemoglobin; MCH: mean corpuscular hemoglobin; RDW: relative distribution width; MCV: mean corpuscular volume.

	RBC (*10 ¹² /L)	Hb (mM)	MCH (fmol)	RDW (%)	MCV (fL)
Dam #1 (CD)	10.22	9.8	0.96	23.9	51
Dam #2 (FOD)	7.25	6.6	0.91	28.3	45
Dam #3 (FOD)	8.37	7.4	0.88	30.6	45

Th1 polarization assay

Figure S2. Effect of perinatal fish oil diet (FOD) on *ex vivo* Th1 polarization of naïve CD4⁺ T cells. mRNA expression of *Ifng* (a), *Il10* (B) and *Tbx21* (C) were measured at 0, 4, 5 and 6 days after incubation of naïve CD4⁺ T cells in presence of α -CD3, α -CD28, IL-2 and IL-12. Splenic naïve CD4⁺ T cells were isolated at the end of lactation from offspring after dams received a control diet (CD) or FOD during gestation and lactation. Data are presented as boxplots. Statistical analysis by linear mixed-effects model. $n = 32$ -35 vs. 25-27 (CD vs. FOD).

Th2 polarization assay

Figure S3 (continued on next page).

Figure S3. Effect of perinatal fish oil diet (FOD) on ex vivo Th2 polarization of naïve CD4⁺ T cells. Extracellular IFN- γ (A), and mRNA expression of *Il4* (B), *Il5* (C), *Ifng* (D), *Il10* (E), *Tbx21* (F) and *Foxp3* (G) were measured at 0, 4, 5 and 6 days after incubation of naïve CD4⁺ T cells in presence of α -CD3, α -CD28, IL-2 and IL-4. Splenic naïve CD4⁺ T cells were isolated at the end of lactation from offspring after dams received a control diet (CD) or FOD during gestation and lactation. Data are presented as boxplots. Statistical analysis by linear mixed-effects model. n = n = 27-32 vs. 20-25 (CD vs. FOD).

Chapter 5

Maternal fatty acid status during pregnancy versus offspring inflammatory markers: a canonical correlation analysis of the MEFAB cohort

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Abstract

The development of inflammatory lung disorders in children is related to maternal diet during pregnancy. We therefore aimed to examine maternal fatty acid (FA) status and its associations with inflammatory markers and lung conditions in the child. This was done by analyzing data from the MEFAB cohort using multivariate canonical correlation analysis (CCA). In the MEFAB cohort, maternal plasma phospholipid FAs were measured at 16, 22 and 32 weeks of pregnancy, and at day of birth. Child inflammatory markers and self-reported doctor diagnosis of inflammatory lung conditions were assessed at 7 years of age. Using CCA, we found that maternal FA levels during pregnancy were significantly associated with child inflammatory markers at 7 years of age and that Mead acid was the most important FA for this correlation. To further verify the importance of Mead acid, we examined the relation between maternal Mead acid levels at day of birth with the development of inflammatory lung disorders in children. After stratification for the child's sex, maternal Mead acid levels at day of birth were significantly related with self-reported doctor diagnosis of asthma and lung infections in boys, and bronchitis and total number of lung conditions in girls. Future studies should investigate whether the importance of Mead acid in the relation between maternal FA status and inflammation and lung conditions in the child is due to its role as biomarker for essential fatty acid deficiency or due to its own biological function as pro-inflammatory mediator.

Introduction

Inflammatory lung diseases are a major burden to global health. According to the World Health Organization (WHO), respiratory infections are the fourth leading cause of both death and burden of disease in the world [1]. Similarly, asthma prevalence has increased over the past few decades [2-4], making asthma the most common chronic disease in children [5]. The development of inflammatory lung disorders in children has been related to changes in maternal diet during pregnancy. Animal studies have demonstrated that changes in maternal diet during gestation predispose weanling offspring to airway inflammation, airway hyperresponsiveness, and increased susceptibility to Respiratory Syncytial Virus (RSV) infection [6,7]. Likewise, both observational and intervention studies in humans have shown that the risk of having asthma and asthma-related symptoms in offspring is negatively correlated with maternal intake of n-3 polyunsaturated fatty acids (PUFAs) [8-13]. Furthermore, maternal fatty acid (FA) status during pregnancy has been linked to inflammation in general and the development of the immune system in the child [14].

When examining maternal FA status during pregnancy and its relation with offspring health, FA levels are often estimated through the use of food frequency questionnaires (FFQs) [9,15], rather than using objective FA biomarkers, such as plasma phospholipids. Furthermore, studies usually assess maternal FAs at a single prenatal time point [16,17], instead of measuring them throughout pregnancy. Moreover, the relation between maternal FA status and offspring health is commonly studied by analyzing single associations between an individual exposure variable and an individual outcome parameter [9,15-17]. However, the complex etiology of biological disorders may be better studied by examining a set of exposure variables and a set of outcome parameters simultaneously [18].

The aim of this study was to examine maternal FA status and its associations with inflammation and lung conditions in the child, while averting the aforementioned shortcomings. This was done by analyzing data from the Maastricht Essential Fatty Acid Birth (MEFAB) cohort using canonical correlation analysis (CCA). The MEFAB cohort was established to study the associations between maternal FA status during pregnancy and pregnancy outcomes [19]. In this cohort, maternal plasma phospholipid FAs were measured at 16, 22 and 32 weeks of pregnancy, and at day of birth, and child inflammatory markers were measured at 7 years of age. CCA is a statistical model which assesses the multivariate correlation between two sets of variables. Although used in many research areas, such as social and behavioral research [20], bioinformatics [21], and genetics [22], CCA has not been used to study the relation between maternal fatty acid status and child inflammation before.

This paper shows that maternal plasma FA levels during pregnancy are significantly associated with child inflammatory markers at 7 years of age and that the FA Mead acid is most important for this correlation. In addition, we show that maternal Mead acid levels at day of birth are significantly correlated with self-reported doctor diagnosis of various inflammatory lung conditions in the child.

Methods

A schematic overview of the study design and analysis workflow are presented in Figure 1.

Study design, setting and participants

Pregnant women attending one of three antenatal clinics in the south the Netherlands between 1989 and 1995 were asked to participate in the cohort. Women were eligible for participation if they were less than 16 weeks pregnant and did not suffer from any cardiovascular, neurological, renal or metabolic condition. Originally, 1203 women were included in the MEFAB cohort. For the follow-up, 305 singletons born between 1990 and 1994 were included between 1997 and 2000. For the current analysis, only mother-child pairs were included for which there were no missing data in any of the maternal plasma fatty acid concentrations or child plasma inflammatory markers, leading to an n of 173

Figure 1. Schematic overview of the study design and analysis workflow. Wk, weeks of pregnancy; FAs, fatty acids; PCA, principle component analysis; CCA, canonical correlation analysis.

mother-child pairs. The study was approved by the medical ethics committee of the University hospital Maastricht and Maastricht University. Written consent was given by pregnant women before enrolment in the initial study and again by both parents (if possible) for the follow-up study.

Exposure variables

To measure maternal FA status during pregnancy, maternal venous blood samples were collected in EDTA tubes around 16, 22 and 32 weeks of pregnancy, and immediately after delivery. Concentrations of 39 different plasma phospholipid FAs were determined by capillary gas-liquid chromatography as described previously [23] and expressed in mg/L. See Table S1 for a list of the FAs that were measured.

Outcome variables

At seven years of age, offspring inflammatory markers were measured in plasma. These markers included: total white blood cell, monocyte, granulocyte and lymphocyte count ($\times 10^9$ /L) determined by Beckman-Coulter Gen-s (Beckman Coulter, Brea, CA, USA); tissue plasminogen activator (tPA) concentration (ng/ml) and enzyme activity (IU/ml) measured by ELISA and bioimmunoassay, respectively (Biopool International, Ventura, CA, USA); plasminogen activator inhibitor-1 (PAI-1) concentration (ng/ml); leptin concentration (μ g/l) determined by RIA (Linco Research, St Charles, MO, USA); fibrinogen concentration (g/l) measured by the Clauss method [24]; and C-reactive protein (CRP) concentration (mg/l) assayed with an in-house ELISA using polyclonal antibodies as catching and tagging antibodies labelled with HRP (Dako, Glostrup, Denmark).

To assess the children's history of lung conditions, their parents were asked at follow-up whether their child was ever diagnosed by a medical doctor with asthma, bronchitis, or pneumonia which was treated with antibiotics.

Confounding variables

Information on potential confounders was obtained using hospital records for maternal age (years) and BMI (kg/m^2) at study entry, duration of pregnancy (days), child sex, and child birthweight (grams). Information regarding maternal smoking and alcohol consumption during pregnancy (yes or no), breastfeeding (yes or no) with or without a combination of formula feeding, presence of pets (dog, cat, rodent or bird) at home during childhood (yes or no), and daycare attendance during first four years (yes or no) was obtained from

questionnaires. Child BMI at seven years of age (kg/m^2) was assessed by a nurse during a follow-up visit.

Canonical correlation analysis

The relation between maternal FA status and child inflammatory status was studied using CCA. CCA is a method which explores the linear relationship between two multivariate data sets X and Y (equation 1). CCA does this by making separate linear combinations (called canonical variates) of both data sets by multiplying each separate variable in X or Y with a coefficient (a so-called canonical weight) and taking the sum of these products (equation 2). Canonical weights are chosen in such a way that the correlation between the canonical variates of X and Y is maximized. CCA develops as many pairs of canonical variates as there are variables in the smallest of the two data sets. Furthermore, all canonical variates are orthogonal (independent) of each other, to make sure that each pair of canonical variates represents a different relationship between X and Y . To test if the correlation between two canonical variates is significant, an asymptotic test using F-approximation of Wilks' Lambda was applied. Only canonical variate pairs with a significant correlation were used for biological interpretation. Individual variables were considered to have a considerable contribution to a canonical correlation if their canonical loadings, which represent the correlation between the individual variable and the conical variate, were $>|0.3|$.

$$X = \begin{pmatrix} x_1 \\ x_2 \\ \dots \\ x_p \end{pmatrix} \quad Y = \begin{pmatrix} y_1 \\ y_2 \\ \dots \\ y_q \end{pmatrix} \quad (1)$$

$$\begin{aligned} U_1 &= a_{11}x_1 + a_{12}x_2 + \dots + a_{1p}x_p & V_1 &= b_{11}y_1 + b_{12}y_2 + \dots + b_{1p}y_p \\ U_2 &= a_{21}x_1 + a_{22}x_2 + \dots + a_{2p}x_p & V_2 &= b_{21}y_1 + b_{22}y_2 + \dots + b_{2p}y_p \\ &\dots & &\dots \\ U_p &= a_{p1}x_1 + a_{p2}x_2 + \dots + a_{pp}x_p & V_p &= b_{p1}y_1 + b_{p2}y_2 + \dots + b_{pp}y_p \end{aligned} \quad (2)$$

Equation 1 and 2. Canonical correlation analysis explores the linear relationship between two multivariate data sets X and Y (equation 1). It does so by making separate linear combinations (canonical variates) of both data sets by multiplying each separate variable in X or Y with a canonical weight and taking the sum of these products (equation 2).

Model optimization

First a full CCA model was obtained using all 39 maternal fatty acids as independent variables and all child inflammatory markers as dependent variables (Figure 2A). To further identify which specific fatty acids were most important for this relation, the number of independent variables in the model was reduced by deleting the fatty acid variable with the

Figure 2. Analysis strategy for performing canonical correlation analysis (CCA), reducing the number of independent variables, obtaining an optimal model, and selecting the most important variables. A) A full CCA model was obtained, containing all 39 maternal fatty acids vs. all child inflammatory markers. B) To reduce the number of independent variables in the CCA model, the fatty acid variable with the lowest absolute, standardized weight was eliminated and the analysis was repeated. C) The process of removing the least contributing independent variable and rerunning the analysis was iterated until an optimum was achieved between a low number of variables and a high correlation. This optimum was set at the point where the canonical correlation coefficient (r) starts decreasing with increasing intervals while cutting out independent variables (indicated by the red dot). D) With the optimal number of independent variables, an optimal CCA model was made. E-F) Fatty acids and inflammatory markers with an absolute loading >0.3 (cut-off indicated by the red line) were selected as most important for the canonical correlation.

smallest contribution to the model (i.e. with the smallest absolute, standardized canonical weight) and the analysis was repeated (Figure 2B). By removing variables, information is withdrawn from the model and the correlation decreases. Therefore, this process of removing the least contributing independent variable was iterated until an optimal balance was achieved between a low number of independent variables and a high correlation (Figure 2C). This optimum was chosen to be at the point where the rate of decrease in correlation due to a diminishing number of independent variables suddenly increased, i.e. where the correlation coefficient suddenly started dropping quicker. With this optimal number of independent variables, an optimal CCA model was made (Figure 2D). For all optimized CCA models, the most important maternal fatty acids and child inflammatory markers were identified by selecting variables with an absolute canonical loading $>|0.3|$ (Figure 2E-F).

Logistic regression

The relation between maternal FA variables with a canonical loading $>|0.3|$ and offspring lung disorders was assessed using binomial and ordinal logistic regression. The influence of potential confounders on logistic regression was determined by including the potential confounder as a covariate in the analysis. If inclusion of the confounder led to a significant covariate effect, the main effect not being significant anymore, or a change $>10\%$ of the main effect odds ratio, the variable was considered to be a confounder. For all statistical analyses, $p < 0.05$ was considered significant. All statistical analyses were performed using R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). CCA was done with CCA package version 1.2.

Results

Population characteristics

Practically all mothers included in the analysis were Caucasian (99%; Table 1). On average, mothers were 30 years-old when entering the study and had a BMI of 24 kg/m^2 and a normal pregnancy duration (279 days). Twenty percent of the mothers smoked during pregnancy, with a mean of nine cigarettes per day. Three percent consumed alcohol during pregnancy, but none specified how much. Deliveries were mostly vaginal (95%) and half of the infants were breastfed for some period of time (47%). About half of the children were female (43%). On average, children had a normal birthweight (3362 grams) and BMI at seven years-of-age (15 kg/m^2).

Table 1. Population characteristics of the MEFAB participants included in this study (n = 173 mother-child pairs). SD, standard deviation; IQR, interquartile range.

Variable	Mean (SD)	Median (IQR)
Mother		
Ethnicity (Caucasian %)	99.41	
Age at entry study (years)	30.12 (4.14)	29.54 (5.33)
BMI at entry study (kg/m ²)	23.56 (3.75)	23.05 (3.66)
Pregnancy		
Duration (days)	279 (11)	281 (12)
Smoking (%)	19.77	
Cigarettes per day	9.17 (5.62)	9.50 (5.00)
Alcohol consumption (%)	2.92	
Caesarian section (%)	4.62	
Breastfeeding (%)	46.82	
Child		
Sex (Female %)	43.35	
Birthweight (grams)	3362 (495)	3400 (640)
BMI at 7 years	15.47 (1.88)	15.03 (2.12)
Asthma (%)	6.98	
Bronchitis (%)	22.22	
Lung infection (%)	7.06	

Maternal plasma fatty acid status changes throughout pregnancy

To assess the progression of maternal fatty acid status during pregnancy, PCA was applied to maternal plasma concentrations of 39 different FAs during pregnancy. The result from this is shown in Figure 3, with each dot representing a mother, with different colors representing different prenatal time points and the position of the dot representing the overall FA status. The gradual shift of the cloud of dots from week 16 to week 22, to week 32, to partus indicates that the overall maternal FA profile gradually changed throughout pregnancy, with the largest shift occurring between 16 and 22 weeks of pregnancy.

Maternal fatty acid status during pregnancy correlates with child inflammatory status at seven years of age

To assess the multivariate relation between maternal FA levels during pregnancy and inflammatory markers in the child at seven years of age, CCA was used. Since PCA showed that the maternal fatty acid status gradually changed throughout pregnancy, a separate CCA

Figure 3. Principle component analysis of 39 maternal plasma fatty acid concentrations, measured at 16, 22 and 32 weeks of pregnancy, and at day of birth (partus). Data are presented as scatter plot of the first and second principle component (PC), covering 15% and 5%, respectively, of the total variance, with each dot representing one mother at a specific gestational time point ($n = 173$ mothers).

was performed for each prenatal time point. The optimized CCA models for week 16, week 22 and week 32 of pregnancy and partus contained sixteen, fourteen, fifteen and nineteen independent FA variables, respectively (Table 2). For all these optimized CCA models, the first canonical variates represented significant, moderate canonical correlations (week 16: $r=0.60$, $p<0.001$; week 22: $r=0.62$, $p<0.001$; week 32: $r=0.64$; $p=0.001$; partus: $r=0.61$, $p=0.028$; Table 2). All lower-order canonical variates were not significant and thus not used for further biological interpretation (Table S2). For each significant canonical variate, the most important independent and dependent variables were identified by selecting variables with an absolute canonical loading >0.3 . Of these various fatty acids and inflammatory markers that were identified to be important for the different canonical correlations, Mead acid (20:3n9) and granulocyte count, respectively, occurred most often (Table 2).

Maternal Mead acid levels at day of birth are positively associated with the development of lung conditions in offspring

Since Mead acid occurred most often as important fatty acid variable in the CCA, the relevance of maternal Mead acid levels during pregnancy was further assessed by examining the association between maternal plasma levels of Mead acid at day of birth and the odds of the child developing asthma, bronchitis or lung infection during the first seven years of age. Mead acid data from day of birth were used, since Mead acid concentrations

Table 2. Canonical correlation analysis of maternal plasma fatty acid (FA) concentrations at 16, 22 and 32 weeks of pregnancy, and at day of birth (partus) versus child plasma inflammatory markers at seven years of age (n = 173 mother-child pairs). r, canonical correlation coefficient.

	No. of FAs in CCA model	r	p-value	FAs loading >0.3	Inflammatory markers loading >0.3
Week 16	16	0.60	<0.001	24:1n9 18:1DMA	Monocytes Granulocytes tPA activity
Week 22	14	0.62	<0.001	20:3n9	White blood cells Granulocytes PAI-1 Leptin
Week 32	15	0.64	0.001	22:2n6 16:0DMA 20:3n9	White blood cells Granulocytes tPA activity tPA concentration PAI-1
Partus	19	0.61	0.028	20:3n9 24:1n9 22:2n6 16:0DMA	White blood cells Granulocytes tPA concentration PAI-1 CRP

Figure 4. Mead acid concentrations in maternal plasma at 16, 22 and 32 weeks of pregnancy, and at day of birth (partus). Data are presented as boxplots, with dots representing values higher than 1.5 times the interquartile range above the third quartile (n = 173 mothers).

increased throughout pregnancy, with the highest concentrations and the largest variation occurring at that specific time point (Figure 4). Using unadjusted binomial regression, a significant, positive relation was found between maternal Mead acid levels and self-

reported doctor diagnosis of bronchitis (OR [95% CI] = 1.17 [1.01 – 1.34]) and lung infection (OR [95% CI] = 1.27 [1.04 – 1.55]), but not asthma (Table 3). Furthermore, unadjusted ordinal logistic regression showed a significant, positive association between maternal Mead acid concentrations at day of birth and the total number of self-reported doctor-diagnosed lung conditions that the child suffered from during the first seven years of age (OR [95% CI] = 1.18 [1.03 – 1.34]; Table 3). Adjusting the logistic regression models for maternal age or BMI at study entry, gestational age, smoking or alcohol use during pregnancy, birthweight, breastfeeding, presence of pets, daycare attendance, or child BMI at seven years of age left the effect estimate and significance of Mead acid largely unchanged (Table S3). Stratification for child sex, however, did lead to sex-specific results. For asthma, bronchitis and number of lung conditions, associations were significant for girls (OR [95% CI] = 1.77 [1.09 – 3.62], 1.32 [1.03 – 1.72], and 1.40 [1.10 – 1.80], respectively) but not boys, whereas for lung infection, associations were significant for boys (OR [95% CI] = 1.28 [1.02 – 1.63]), but not girls (Table 3).

Table 3. Associations between maternal plasma mead acid concentration at day of birth and self-reported doctor diagnosis of lung conditions in the child during the first seven years of age (n = 173 mother-child pairs). Statistical analysis by binomial logistic regression (for asthma, bronchitis and lung infection) or ordinal logistic regression (for number of lung conditions). OR (odds ratio), 95% CI (confidence interval) and p-value are shown for both unadjusted results and results stratified for child sex.

	OR	95% CI	p-value
Asthma			
Unstratified	1.13	0.91 – 1.38	0.228
Girls	1.77	1.09 – 3.62	0.038
Boys	1.03	0.77 – 1.29	0.842
Bronchitis			
Unstratified	1.17	1.01 – 1.34	0.030
Girls	1.32	1.03 – 1.72	0.029
Boys	1.11	0.93 – 1.33	0.227
Lung infection			
Unstratified	1.27	1.04 – 1.55	0.014
Girls	1.27	0.82 – 1.91	0.237
Boys	1.28	1.02 – 1.63	0.029
Number of lung conditions			
Unstratified	1.18	1.03 – 1.34	0.007
Girls	1.40	1.10 – 1.80	0.003
Boys	1.12	0.94 – 1.31	0.093

Discussion

The development of inflammatory lung disorders has previously been shown to be related to maternal diet during pregnancy. However, estimates of prenatal exposures and statistical analysis were often suboptimal in previous studies. Therefore, this study examined the relation between maternal FA status and child inflammation by analyzing data from the MEFAB cohort using CCA. This paper showed that maternal plasma FA levels during pregnancy were significantly associated with child inflammatory markers at 7 years of age and that Mead acid was the most important FA for this correlation. In addition, we showed that maternal Mead acid levels at day of birth were significantly correlated with self-reported asthma, bronchitis and the total number of lung conditions in girls, and with self-reported lung infection in boys.

First, we showed that the overall profile of maternal FAs gradually changed throughout pregnancy. This finding emphasizes the importance of assessing FA levels at consistent prenatal time points when studying the relation between maternal FA status and offspring health, rather than assessing FA exposure at varying prenatal time points, as has been the case for previous studies. For instance, Notenboom et al. measured maternal FA levels in the 34th to 36th week of gestation [16], whereas Rucci et al. measured maternal FA levels around the 20th week of gestation [17], making comparison of both studies suboptimal. Moreover, Miyake et al. assessed maternal FA consumption at study entry, irrespective of the gestational age [9], thereby potentially introducing heterogeneity in the exposure data set. The current study, in contrast, measured maternal FA levels at 16, 22 and 32 weeks of gestation, and at day of birth, thereby covering both the second and third trimester. In addition, maternal FA status was assessed by measuring plasma phospholipid FAs, which represent FA levels of the past few days [25]. Altogether, the exposure variables as assessed in the current study may be considered a comprehensive representation of prenatal maternal FA status.

Using CCA, we examined the multivariate relation between child inflammatory markers at 7 years of age and maternal plasma FA levels at 16, 22 and 32 weeks of gestation, and at day of birth. With this approach, we found that for all prenatal time points, maternal FA status was moderately, but significantly associated with child inflammatory markers at 7 years of age. In addition, Mead acid was identified most often as an important exposure variable for these multivariate correlations. Mead acid (20:3n-9) is an n-9 PUFA which is synthesized *in vivo* from oleic acid (18:1n-9) through elongation and desaturation by the enzymes Fatty acid desaturase 1 (Fads1), Fatty acid desaturase 2 (Fads2) and Elongation of very long chain fatty acids protein 5 (Elovl5) [26]. Fads1, Fads2 and Elovl5 also elongate and desaturate the n-6 and n-3 PUFAs linoleic acid (LA, 18:2n-6) and α -linolenic acid (ALA, 18:3n-

3) into their respective longer-chain derivatives, such as arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) [27]. As a result of these shared metabolic pathways between n-3, n-6 and n-9 PUFAs, Mead acid synthesis is enhanced when n-3 and n-6 PUFA levels are low, making Mead acid a biomarker for essential fatty acid deficiency [28]. Next to this, Mead acid has its own biological functions. Similar to the conversion of AA into 5-hydroxyeicosatetraenoic acid (5-HETE) by 5-lipoxygenase (5-LOX) and its subsequent oxidation into 5-oxo-eicosatetraenoic acid (5-oxo-ETE) by 5-hydroxyeicosanoid dehydrogenase (5-HEDH), Mead acid is metabolized into 5-hydroxyeicosatrienoic acid (5-HETrE) and 5-oxo-eicosatrienoic acid (5-oxo-ETrE), respectively, by the same enzymes [29,30]. In accordance with these structural analogies, 5-oxo-ETrE is as potent as 5-oxo-ETE in inducing chemotaxis, surface expression of CD11b and calcium mobilization in neutrophils, as well as increasing actin polymerization in eosinophils [29]. Mead acid thus acts as a pro-inflammatory mediator, thereby potentially actively contributing to the course of inflammatory lung conditions, such as lung infections, asthma and bronchitis.

Next to Mead acid being identified as an important exposure variable, the most frequently identified important outcome variable for the multivariate correlation between maternal FA status and child inflammation was granulocyte count. Granulocytes play an important role in various inflammatory lung conditions. For instance, both eosinophils and neutrophils are associated with clinical severity and pulmonary dysfunction in asthma patients [31-34]. Similarly, fungal infection of the lungs is associated with pulmonary influx of eosinophils [35], whereas neutrophils contribute to early defense against pulmonary infection with bacteria [36]. Considering the identification of both Mead acid and granulocyte count as important variables, and given the biological effects Mead acid can have on eosinophils and neutrophils, and the role these granulocytes play in inflammatory lung conditions, Mead acid may possibly exert any potential influence on lung conditions through its effect on granulocytes. Whether this hypothesis is correct and what molecular or cellular mechanisms would be involved in this, remains to be examined by future studies.

To further evaluate the relevance of maternal Mead acid levels during pregnancy, the association between maternal plasma levels of Mead acid at day of birth and the self-reported doctor diagnosis of lung conditions in children was examined. As a result, maternal Mead acid levels at day of birth were positively associated with the diagnosis of several lung conditions in children. After stratification for sex, these associations were significant for asthma, bronchitis and number of lung infections for girls but not boys, whereas for lung infection, associations were significant for boys but not girls. These sex-specific associations may be related to known differences between males and females regarding development of the lungs and lung disorders. For instance, boys are more likely to develop asthma than

girls, whereas adult females have a higher prevalence than adult males [37], suggesting the involvement of sex hormones. Furthermore, neonate boys have a higher chance of mortality due to respiratory distress syndrome than neonate girls [38]. Next to males and females having different lung development, differences may also occur in the way male and female offspring respond to changes in maternal diet during pregnancy, as we have shown previously [39]. Future studies will have to confirm whether indeed the relation between maternal FA status and offspring lung conditions differ between male and female offspring, and if so, how these changes occur.

When interpreting results from the current study, it should be considered that the MEFAB cohort comprises a relatively healthy population. Although this consistency within the cohort enhances the comparability within participants, it also limits the applicability of the findings to less healthy populations, such as people with obesity. Furthermore, the MEFAB cohort has a relatively homogeneous genetic background, with 99% of the participants in the current analysis being Caucasian. Given the differences that exist in inflammatory markers between ethnicities [40,41], the associations reported in this study may thus have been different if the ethnic background of the population would have been more diverse. Moreover, solely studying Caucasian participants in biomedical research contributes to health disparity. Future studies should therefore include other ethnicities as well, as did Rucci et al., who's Generation R cohort consisted of 66% Europeans and 34% non-Europeans [17].

In conclusion, our findings support the previously reported relation between inflammatory lung conditions and maternal diet during pregnancy. Furthermore, this work demonstrated the application of CCA within this field of research, which may lead to future studies using a similar approach. Lastly, future studies should investigate whether the importance of Mead acid in the relation between maternal FA status and child inflammation is due to its role as biomarker for essential fatty acid deficiency, due to its own biological function as pro-inflammatory mediator, or due to a combination of both.

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Supplement

Table S1. List of the 39 fatty acids that were measured in maternal plasma at 16, 22 and 32 weeks of pregnancy, and at day of birth. DMA – dimethyl acetal, tr – truncated.

Saturated	n-7	n-9	n-6	n-3	Derivatives
14:0	16:1n-7	18:1n-9	18:2n-6	18:3n-3	18:0 DMA
15:0	18:1n-7	18:2n-9	18:3n-6	20:3n-3	18:1 DMA
16:0	20:1n-7	20:1n-9	20:2n-6	20:4n-3	16:1n-7 tr
17:0		20:3n-9	20:3n-6	20:5n-3	
18:0		22:1n-9	20:4n-6	22:3n-3	
20:0		22:3n-9	22:2n-6	22:5n-3	
22:0		24:1n-9	22:4n-6	22:6n-3	
23:0			22:5n-6		
24:0			24:2n-6		

Table S2. Canonical variates of canonical correlation analysis of maternal plasma fatty acid (FA) concentrations at 16, 22 and 32 weeks of pregnancy, and at day of birth (partus) versus child plasma inflammatory markers at seven years of age (n = 173 mother-child pairs). r, canonical correlation coefficient; p, p-value.

Canonical variate	Week 16		Week 22		Week 32		Partus	
	r	p	r	p	r	p	r	p
1	0.60	<0.001	0.62	0.000	0.64	0.001	0.61	0.028
2	0.45	0.108	0.48	0.194	0.44	0.431	0.46	0.594
3	0.43	0.305	0.41	0.675	0.41	0.759	0.43	0.823
4	0.38	0.588	0.35	0.918	0.35	0.943	0.41	0.951
5	0.34	0.785	0.32	0.983	0.33	0.983	0.35	0.995
6	0.33	0.893	0.22	0.998	0.25	0.998	0.28	0.999
7	0.25	0.980	0.19	0.997	0.23	0.999	0.26	0.999
8	0.22	0.982	0.16	0.994	0.16	1.000	0.21	0.999
9	0.18	0.984	0.14	0.984	0.10	1.000	0.18	0.997
10	0.09	0.987	0.08	0.967	0.08	0.989	0.14	0.982

Table S3. Associations between maternal plasma mead acid concentration at day of birth and self-reported doctor diagnosis of lung conditions in the child during the first seven years of age, adjusted for various population characteristics (n = 173 mother-child pairs). Statistical analysis by binomial logistic regression (for asthma, bronchitis and lung infection) or ordinal logistic regression (for number of lung conditions). OR, odds ratio; 95% CI, 95% confidence interval.

	OR	95% CI	p-value
Asthma			
Unadjusted	1.13	0.91 – 1.38	0.228
Maternal age	1.13	0.91 – 1.38	0.241
Maternal BMI	1.16	0.93 – 1.42	0.166
Gestational age	1.11	0.88 – 1.37	0.328
Smoking pregnancy	1.13	0.90 – 1.38	0.242
Alcohol pregnancy	1.13	0.90 – 1.37	0.260
Birthweight	1.13	0.90 – 1.38	0.241
Breastfeeding	1.15	0.92 – 1.40	0.198
Pets	1.13	0.91 – 1.38	0.217
Daycare attendance	1.13	0.91 – 1.38	0.235
Child BMI	1.12	0.90 – 1.37	0.265
Lung infection			
Unadjusted	1.27	1.04 – 1.55	0.014
Maternal age	1.31	1.07 – 1.62	0.009
Maternal BMI	1.27	1.04 – 1.55	0.016
Gestational age	1.26	1.04 – 1.54	0.018
Smoking pregnancy	1.27	1.04 – 1.55	0.014
Alcohol pregnancy	1.29	1.06 – 1.58	0.013
Birthweight	1.27	1.04 – 1.55	0.015
Breastfeeding	1.26	1.03 – 1.54	0.018
Pets	1.27	1.04 – 1.55	0.014
Daycare attendance	1.27	1.04 – 1.54	0.016
Child BMI	1.27	1.04 – 1.54	0.017
Bronchitis			
Unadjusted	1.17	1.01 – 1.34	0.030
Maternal age	1.17	1.02 – 1.35	0.024
Maternal BMI	1.18	1.03 – 1.37	0.020
Gestational age	1.15	1.00 – 1.32	0.043
Smoking pregnancy	1.19	1.04 – 1.38	0.013
Alcohol pregnancy	1.20	1.04 – 1.38	0.013
Birthweight	1.16	1.01 – 1.34	0.034
Breastfeeding	1.17	1.02 – 1.35	0.024
Pets	1.17	1.01 – 1.34	0.030

Daycare attendance	1.16	1.01 – 1.34	0.034
Child BMI	1.15	1.00 – 1.33	0.043
Number of lung conditions			
Unadjusted	1.18	1.03 – 1.34	0.007
Maternal age	1.19	1.04 – 1.36	0.005
Maternal BMI	1.20	1.05 – 1.37	0.004
Gestational age	1.17	1.02 – 1.33	0.010
Smoking pregnancy	1.20	1.05 – 1.38	0.003
Alcohol pregnancy	1.21	1.05 – 1.38	0.003
Birthweight	1.18	1.03 – 1.34	0.006
Breastfeeding	1.19	1.04 – 1.35	0.006
Pets	1.18	1.03 – 1.35	0.007
Daycare attendance	1.18	1.03 – 1.34	0.008
Child BMI	1.17	1.03 – 1.34	0.009

Chapter 6

General discussion

Introduction

The aim of this thesis was to study the perinatal effects of maternal dietary fat on molecular responses in offspring immunologic, metabolic and pulmonary health. In **chapter 2**, we showed that a perinatal high-fat diet altered offspring hepatic gene expression and DNA methylation of pathways involved in lipid metabolism, oxidative stress response, and cell proliferation. Furthermore, we demonstrated in **chapter 3** that acute ozone exposure increased pulmonary oxidant status and upregulated the molecular control of mitophagy, and that in female offspring these responses were exacerbated by a perinatal high-fat diet. In **chapter 4**, a perinatal fish oil diet not only reduced *ex vivo* cytokine levels of polarizing CD4⁺ T cells, but also induced anemia with microvesicular hepatic steatosis in mouse offspring. Finally, in **chapter 5**, we showed that maternal fatty acid status during pregnancy was associated with child inflammatory markers at seven years of age, and that maternal Mead acid levels correlated with the development of inflammatory lung conditions in the child. In order to value the findings presented in this thesis and to extrapolate clinical implications from them, a number of topics, such as the timing of exposure, the influence of sex effects, the use of animal models, and future perspectives will be considered and evaluated in this general discussion.

Timing of exposure

The association or effect between a maternal dietary exposure and offspring health may be influenced by the timing of that specific exposure. Van Vlies et al. fed dams a high n-3 polyunsaturated fatty acid (PUFA) diet either during gestation, or during lactation, or during both gestation and lactation [1]. All three approaches led to an enhanced Th1-mediated immune response, as well as an attenuated Th2-mediated allergic response compared with control animals. However, the high n-3 PUFA diet was significantly more effective during lactation than during gestation alone or during gestation and lactation together. Similarly, immune cell populations in the thymus and in the spleen were significantly affected in offspring exposed to the high n-3 PUFA diet during lactation or during both gestation and lactation, compared with control, but not in offspring exposed to this diet only during gestation. These findings suggest that in mice the positive effects of supplementation with n-3 PUFAs during early life may be most beneficial during lactation, rather than during gestation. The Dutch famine studies, which examined the relation between prenatal exposure to undernutrition and health effects in adult offspring, distinguished between exposure during early, mid, or late gestation. They showed that multiple adverse health effects, such as high BMI, atherogenic lipid profile, disturbed blood coagulation profile, elevated blood pressure-response to stress, and prevalence of coronary heart disease, were all associated with famine exposure during early gestation, but not with famine exposure

during mid or late gestation [2-6]. Conversely, prevalence of microalbuminuria and prevalence of obstructive airways diseases in adults were associated with famine exposure during mid gestation, but not with famine exposure during early or late gestation [7,8]. Lastly, glucose tolerance was decreased in adult offspring exposed to famine during any stage of gestation [9,10]. Taken together, these findings may imply that the window of greatest susceptibility not only depends on the specific exposure, but also on the associated health outcome.

In this thesis, the influence of the timing of exposure was examined in **chapter 5**. In this chapter, we showed that even without an intervention, a prenatal exposure may vary over time, as was indicated by the gradual change in maternal plasma fatty acid profile throughout pregnancy in mothers of the MEFAB cohort. Despite these differences in exposure between the prenatal time points (i.e. weeks 16, 22 and 32 of pregnancy, and day of birth), significant multivariate correlations were found between maternal plasma fatty acid status and offspring inflammatory health for all examined prenatal time points. In **chapter 2** and **chapter 3** of this thesis, dams were fed a high-fat diet starting from six weeks before gestation until the end of lactation. In **chapter 4**, dams received a fish oil diet during gestation and lactation. For these three chapters, the effects of a different window of exposure were not studied. However, considering the influence timing of exposure is known to have on the relation between maternal dietary exposures and offspring health, findings in these chapters may thus have been different if other windows of exposure would have been applied. For future intervention studies, researchers should not only contemplate the perinatal period during which mothers or dams are exposed, but also take the specific timing of exposure into account when comparing their results with literature.

Sex effects

Next to timing of exposure, offspring sex may influence the association or effect between maternal dietary exposure and offspring health. Ornellas et al. showed that a maternal high-fat diet in mice had various detrimental effects on metabolic health in both male and female offspring. However, effects on feed intake, body weight gain, fasting glucose levels, glucose intolerance, plasma triglycerides and plasma insulin were largest in males, whereas females were more affected in terms of adiposity [11]. Snow et al. fed female rats a high-fat diet before mating and during gestation and lactation to examine the effect of a perinatal high-fat diet on ozone susceptibility in offspring [12]. They found that the ozone exposure elicited greater metabolic impact in female than male offspring, and that the maternal high-fat diet further potentiated this effect in female offspring.

In this thesis, the influence of offspring sex on the relation between maternal diet and offspring health was studied in **chapter 3**, **chapter 4** and **chapter 5**. In **chapter 3**, acute ozone exposure increased pulmonary oxidative stress and upregulated the molecular machinery that controls receptor-mediated mitophagy. In female offspring, the perinatal high-fat diet exacerbated these responses, whereas in males, responses were similar between offspring from control diet dams and offspring from high-fat diet dams. In **chapter 5**, the association between maternal mead acid plasma levels at day of birth and the development of lung conditions in offspring was affected by offspring sex as well. After stratification for sex, associations between maternal Mead acid levels and asthma, bronchitis and the total number of lung conditions was significant for girls, but not boys, whereas for lung infection, the association was only significant for boys, but not girls. In **chapter 4**, a maternal fish oil diet affected *ex vivo* polarization of naïve CD4⁺ T cells isolated from offspring spleen. For this study, both male and female offspring were included and statistical analysis were adjusted for offspring sex. However, none of the measured outcomes in offspring showed a significant effect of sex. This may have been because offspring animals were assessed at such a young age that hormonal differences between both sexes were insufficiently developed to induce sex effects [13]. In **chapter 2**, sex effects were not assessed, since only male offspring was included in the experiment. Major contributors to sex dimorphisms are estrogens and androgens [14-17]. In females, these effects depend on the hormonal cycle, making females more prone to variation in specific outcomes. For this reason, often only males are included in biomedical studies. However, for scientific results to be properly translated into clinical practice, future studies should not only include both sexes in studies, but also assess outcomes for both sexes separately, or at least adjust for sex in statistical analyses.

Animal models – Ethics

Many studies use animal models to examine the effects of prenatal diets on offspring health. Major advantages of working with small animals such as rats and mice are their short gestation time and lifespan, relatively inexpensive maintenance costs, control over environmental variability, and the ease of diet manipulation [18]. In the Netherlands, performing an animal experiment is prohibited, unless it can be justified that 1) the scientific gain of the experiment outweighs the harm caused to the animal; and that 2) the research question cannot be answered with any alternative approach. Whether or not an animal study meets these requirements is assessed by the Central Committee Animal Experiments (Centrale Commissie Dierproeven, CCD), which is advised by the Animal Experiment Committee (Dierexperimentencommissie, DEC).

When applying for approval of the study described in **chapter 4**, the Animal Experiment Committee initially judged that both conditions for approval of an animal experiment were not met. For the first condition, the committee based its point of view on the fact that the study revolved around the polarization of CD4⁺ T cells and its role in the anti-atopic effects of maternal supplementation with n-3 PUFAs. The committee argued that atopic diseases are not lethal and that therefore this research was not relevant enough to justify the use of animals. For the second condition, the committee did agree that studying prenatal exposures is impossible *in vitro*. However, they did not see any ethical restrictions to performing the study in humans. In a rebuttal, we challenged the committee's decision on both conditions. For the first condition, we discussed that although this specific study revolved around atopic diseases, the polarization of CD4⁺ T cells is a fundamental physiological process within the adaptive immune system which can be applied to many (also lethal) diseases. In addition, we believe that preventing the development of a disease that significantly affects a patient's quality of life is sufficiently relevant to justify the use of animals, even more since an increasing number of diseases that was previously lethal, is chronic nowadays due to improved treatments (e.g. AIDS). For the second condition, we argued that to perform this research in humans, the study should comply with the Embryo Law, since any intervention applied to a pregnant female also affects the unborn child. The Embryo Law states that research involving an embryo is prohibited, unless 1) the research contributes to diagnostics, prevention or treatment of a severe condition in that specific embryo that is subjected to the treatment; or 2) the objections and risks of the intervention have negligible objections and risks for the embryo. For our study, this first condition would not be met, since the intervention would have been applied to healthy women without any indication for future risk of the development of atopy in their children, which made this a fundamental study. Likewise, this second condition was not met as well. To assess the polarization of naïve CD4⁺ T cells, these cells would have to be isolated from secondary lymphoid organs (such as lymph nodes or the spleen) or from peripheral blood. The inability to access lymphoid organs in a human requires no further clarification. Furthermore, withdrawing a sufficient amount of blood for isolation of naïve CD4⁺ T cells is such a negative experience for a one-year-old child (sensitization to allergens is thought to occur early in life [19]), that the objections and risks would not be negligible. Hence, assessing the research question in humans would be unethical and thus would not be approved. In the end, the committee reconsidered its opinion and granted approval for the animal model as described in chapter 4.

Retrospectively, the maternal fish oil diet as studied in **chapter 4** turned out to have serious adverse effects. The perinatal fish oil diet induced anemia in both dams and offspring, and caused microvesicular hepatic steatosis in offspring. In the discussion section of chapter 4, we discuss that this may be due to the dosage of n-3 PUFAs, which was ten times higher in

our study than in previous clinical studies. Nevertheless, our study demonstrated that a perinatal fish oil diet is able to induce detrimental effects in both mothers and offspring, and thus that future studies, both animal and clinical, should carefully consider their dosage. Besides chapter 4, **chapters 2 and 3** used animal models as well to study the effect of maternal diet on offspring health. For these chapters, the design and execution of the animal model were performed by collaborating research groups, which kindly provided samples in order for us to answer research questions that continued on previous findings of those collaborators [12,20,21]. This strategy prevents the unnecessary use of animals that would otherwise occur if a new experiment was performed instead of reusing samples for subsequent studies.

Altogether, we believe that the advantages and scientific gains obtained from using animal models outweigh the disadvantages related to animal welfare and translation, as long as the balance between these benefits and drawbacks is carefully assessed per individual case by external committees. Nevertheless, researchers should be aware of the differences between model species and humans, and of the implications hereof for translation of findings from animals models to clinical practice.

Animal models – Litter effects

One major challenge in studying effects of maternal diet on offspring health in animals is the use of multiparous species, such as rats and mice [22]. When studying maternal exposures using animal models, the treatment is applied to pregnant females and thus to all unborn offspring animals within that female. Consequently, the litter is the experimental unit, as this is the smallest unit to which a treatment can be randomly assigned. The outcomes, however, are measured in individual offspring animals, since these are of scientific interest. Subsequently assessing each individual offspring animal as a separate experimental unit leads to sample size inflation and would be similar to applying a treatment to a single patient and then measuring its blood pressure in both arms and assuming this represents an n of 2. In addition to this, animals from the same litter are more alike than animals from different litters, causing litter mates to be correlated. However, routine analysis methods such as t-test, ANOVA and linear regression assume individuals to be independent of each other. Ignoring the dependencies that actually exist within animals from the same litter may lead to increased false-positive rate or low power [23]. To prevent this, several experimental approaches can be applied [22]. The simplest option is to randomly select one offspring animal per litter and measure the outcome on that animal. Alternatively, multiple offspring animals per litter can be assessed, after which values are averaged for each litter. Lastly, multiple animals can be measured per litter, after which

statistical analysis is performed using mixed-effects models, which are able to take into account the correlated data structure.

In this thesis, litter effects were handled with different approaches. In **chapter 3**, dams were fed a high-fat diet or a control diet before mating, and during gestation and lactation, to study the effect of a perinatal high-fat diet on ozone susceptibility in offspring. Before offspring was exposed to ozone, four pups per litter were randomly selected and allocated to an exposure group: one female for air exposure, one female for ozone exposure, one male for air exposure, and one male for ozone exposure. By spreading animals from the same litter over different experimental groups, observations were independent, and routine approaches (in this case ANOVA) could be used for statistical analysis. In **chapter 4**, dams received a fish oil diet or a control diet during gestation and lactation to examine the effect of perinatal n-3 PUFAs on offspring CD4⁺ T cells. Outcomes were assessed in all offspring animals, after which data were analyzed with linear mixed-effects models. Advantages of this approach are that no animals are wasted, and that less dams have to be included in the experiment to reach a sufficient sample size compared with when only one offspring per litter is analyzed or when outcomes are averaged per litter. In **chapter 5**, litter effects were not an issue, since only singletons were included in the MEFAB cohort. The only chapter in this thesis for which litter effects were handled suboptimally, is **chapter 2**. In this chapter, dams were fed a high-fat diet or a control diet before mating, and during gestation and lactation. After this, offspring animals were divided over two groups. The first group was assessed at 12 weeks of age, whereas the other was assessed at 28 weeks of age. For the first group, only one animal per litter was selected, thereby preventing issues related to litter effects. For the second group, however, 11 animals were independent (they originated from 11 different litters), whereas the other 7 animals originated from 3 litters. Instead of handling the dependency of observations within this group by analyzing data with mixed-effects models, data were analyzed with a t-test. Although in this particular case the pseudoreplication was minimal compared with an experiment that would have used all litter mates, results may still have been biased to some extent. For future studies, to ensure correct experimental design and statistical analysis of animal models using multiparous species, researchers should be aware of the potential pitfalls these experiments harbor. Herein lies an important role for authorities that review animal experiments, such as the Animal Welfare Body and Animal Experiment Committee. All animal experiments require approval of these authorities, providing the opportunity to prevent issues arising from litter effects when studies are still in their design phase.

Clinical and public health implications

Maternal consumption of a high-fat diet and obesity during pregnancy and lactation predisposes offspring to developing various metabolic disorders, such as obesity and type 2 diabetes, and cardiovascular diseases [24-27]. In **chapter 2**, a perinatal high-fat diet altered hepatic DNA methylation and gene expression of pathways related to lipid synthesis and uptake, oxidative stress response, and cell proliferation. These changes were mechanistically linked to previously reported phenotypic effects of maternal high-fat diet, such as increases in body weight and fat mass, and induction of non-alcoholic fatty liver disease [11,28,29]. These findings provide evidence for the involvement of epigenetics and regulation of gene expression in the effects of prenatal obesity and high-fat diet on offspring metabolic health.

In **chapter 3**, a perinatal high-fat diet exacerbated ozone-induced increases in pulmonary oxidant status and upregulation of the molecular control of mitophagy. These outcomes suggest that mitochondria and oxidative stress play a role in the effect of maternal diet on ozone susceptibility in offspring. Taken together, the results from **chapters 2 and 3** further necessitate the investment in early life nutrition to protect offspring from detrimental metabolic and pulmonary effects.

Clinical studies have previously shown that supplementation with n-3 PUFAs during pregnancy may reduce the risk of developing asthma in children [30-32]. In **chapter 4**, we showed that a perinatal fish oil diet, which contains high amounts of n-3 PUFAs, decreased extracellular cytokine levels of polarizing CD4⁺ T cells. Polarization of CD4⁺ T cells plays an important role in the development of asthma and other atopic diseases [33], and our findings may thus provide evidence for a mechanistic link between maternal n-3 PUFAs and development of atopy in offspring. This mechanistic link, in combination with evidence from observational and clinical studies, corroborates the need for a dietary advise on prenatal consumption and supplementation of n-3 PUFAs. In **chapter 5**, we demonstrated that maternal fatty acid status correlated with child inflammation at seven years of age, and that maternal Mead acid levels were associated with development of inflammatory lung conditions. Considering that Mead acid is a biomarker for essential fatty acid deficiency [34], that Mead acid levels increased throughout pregnancy (**chapter 5**), and that Mead acid metabolites have pro-inflammatory effects [35], these findings further substantiate the need for dietary advise on consumption and supplementation of fatty acids during pregnancy. However, besides positive effects on CD4⁺ T cell polarization, the perinatal fish oil diet in **chapter 4** also induced anemia in both dams and offspring, and caused hepatic microvesicular steatosis in offspring. These findings stress the need to further research potential adverse effects of prenatal supplementation with high levels of n-3 PUFAs on maternal and offspring health. If institutions like the Health Council (Gezondheidsraad) or

the Nutrition Centre (Voedingscentrum) would come to publish an advise for prenatal supplementation with n-3 PUFAs to protect children against asthma or other atopic conditions, upper dosage limits should be carefully considered to prevent adverse effects.

Future perspectives

Based on the topics discussed so far, a number of recommendations may be done for studies yet to come. Firstly, studies that examine specific maternal exposures should compare the effects of different windows of exposure on offspring health. Many animal studies apply an intervention throughout gestation and lactation to ensure that any window of opportunity is not missed. However, by sticking to this approach, it will never be learned which exposure or intervention should be prevented or applied, respectively, during which perinatal phase in order to improve offspring health. Next to this, it would be beneficial if animal and human studies would not only include both sexes, but also analyze them separately. This would improve the translation to new clinical studies and prevent further health disparity between men and women. In addition, future animal studies using multiparous species should take litter effects into account, to prevent the unnecessary introduction of false-positive or false-negative effects, thereby improving the translatability of animal results. Next to these general recommendations, future studies may follow up on findings presented in this thesis. For instance, future studies may examine whether adverse effects of a maternal high-fat diet on offspring metabolism are regulated by changes in offspring gene expression and DNA methylation, as was suggested by the findings from chapter 2. Furthermore, the involvement of oxidative stress and mitochondrial function in the effects of maternal high-fat diet on ozone susceptibility in offspring may be further studied. In addition, future studies should find out whether the adverse effects reported in chapter 4 were indeed caused by high levels of n-3 PUFAs and how these detrimental effects can be prevented in future studies. Lastly, it may be examined whether Mead acid is mechanistically involved in the relation between maternal fatty acid status and inflammatory lung diseases in the child.

Conclusions

The aim of this thesis was to study the perinatal effects of maternal dietary fat on molecular responses in offspring immunologic, metabolic and pulmonary health. In **chapter 2**, we showed that a perinatal high-fat diet altered offspring hepatic gene expression and DNA methylation of pathways involved in lipid metabolism, oxidative stress response, and cell proliferation. Furthermore, we demonstrated in **chapter 3** that acute ozone exposure increased pulmonary oxidant status and upregulated the molecular control of mitophagy, and that in female offspring these responses were exacerbated by a perinatal high-fat diet.

In **chapter 4**, a perinatal fish oil diet not only reduced *ex vivo* cytokine levels of polarizing CD4⁺ T cells, but also induced anemia with microvesicular hepatic steatosis in mouse offspring. In addition, we showed in **chapter 5** that maternal fatty acid status during pregnancy was associated with child inflammation at seven years of age, and that maternal Mead acid levels correlated with the development of inflammatory lung conditions in the child. Finally, in **chapter 6**, the general discussion, we discussed that the association between maternal dietary exposure and offspring health may be influenced not only by the timing of that specific exposure, but also by the sex of the offspring. Next to this, we discussed ethical and practical issues that need to be considered when using animal models for studying the effects of maternal diet on offspring health.

Altogether, this thesis has shown the influence of maternal dietary fat on molecular responses in offspring immunologic, metabolic and pulmonary health. Through these findings, this thesis has further substantiated the importance of maternal diet for later-life health in offspring. Future research should continue to examine mechanisms that underlie both adverse and beneficial effects of maternal nutrition, to further improve the health of future generations.

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Summary

Summary

The fat content and fatty acid composition of the maternal diet during pregnancy and lactation greatly influence the offspring's immunologic, metabolic and pulmonary health. However, the molecular responses and mechanisms that are involved in these effects remain largely unknown. Therefore, this thesis aimed to study the effects of maternal dietary fatty acids and high-fat diet on the molecular responses related to offspring immunologic, metabolic and pulmonary health.

Exposure to a perinatal high-fat diet leads to an impaired metabolic phenotype in mouse offspring. It is less well-known, however, through which mechanisms these long-term effects are transmitted. Therefore, we studied the effect of a combined paternal and maternal high-fat diet on hepatic gene expression and DNA methylation in adult mouse offspring. In **chapter 2**, we showed that this perinatal high-fat diet altered hepatic expression of gene sets involved in lipid metabolism, oxidative stress response and cell proliferation. Furthermore, the perinatal high-fat increased hepatic oxidative stress. In addition, transcription factor binding sites upstream of the metabolic gene *Lpin1* were hypermethylated in offspring livers in response to the perinatal high-fat diet. We hypothesize that these changes underlie the previously reported metabolic disturbances in offspring after exposure to a prenatal high-fat diet.

Next to affecting offspring metabolism, a perinatal high-fat diet is also able to exacerbate ozone-induced adverse effects on lung function, injury and inflammation in offspring, and it has been suggested that mitochondrial dysfunction is involved in this effect. Therefore, the aim of **chapter 3** was to investigate whether a perinatal obesogenic high-fat diet affects ozone-induced changes in offspring pulmonary oxidant status and the molecular control of mitochondrial function. In this study, acute ozone exposure increased pulmonary oxidant status and upregulated the molecular machinery that controls receptor-mediated mitophagy. In female offspring, a perinatal high-fat diet exacerbated these responses, whereas in male offspring, responses were similar for both diet groups. These findings suggest that a perinatal high-fat influences ozone-induced responses on pulmonary oxidant status and the molecular control of mitophagy in female rat offspring.

Maternal supplementation with n-3 polyunsaturated fatty acids (PUFAs) during pregnancy is associated with reductions of asthma features in offspring. The underlying mechanisms, however, are unclear. Since a skewed polarization of CD4⁺ T cells is involved in asthmatic development, we studied the effect of a perinatal n-3 PUFA-rich fish oil diet on *ex vivo* polarization of CD4⁺ T cells in mouse offspring. In **chapter 4**, we showed that a perinatal fish oil diet reduced extracellular cytokine levels of both Th1- and Th2-polarizing CD4⁺ T cells. In

addition, we found that a perinatal fish oil diet induced not only anemia in both dams and offspring, but also in microvesicular steatosis in offspring liver. These results indicate that perinatal exposure to high levels of n-3 PUFAs may have both beneficial and adverse effects in the offspring and thus that dosages should be carefully considered for future studies.

The development of inflammatory lung disorders has previously been shown to be related to maternal diet during pregnancy. However, estimates of maternal fatty acid status and statistical analysis were often suboptimal in previous studies. Therefore, we examined in **chapter 5** the relation between maternal fatty status and child inflammation by analyzing data from the MEFAB cohort using canonical correlation analysis (CCA), a multivariate statistical model. This chapter showed that maternal fatty acid status during pregnancy was significantly associated with child inflammation at seven years of age and that the fatty acid Mead acid was most important for this correlation. In addition, maternal Mead acid levels at day of birth were significantly correlated with the development of inflammatory lung conditions in the child. Future studies should investigate whether the importance of Mead acid in the relation between maternal fatty acid status and child inflammation is due to its role as biomarker for essential fatty acid deficiency, due to its own biological function as pro-inflammatory mediator, or possibly a combination of both.

In summary, this thesis aimed to study the effects of maternal dietary fatty acids and high-fat diet on the molecular responses related to offspring immunologic, metabolic and pulmonary health. We showed that a maternal high-fat diet is able to affect gene expression and DNA methylation in offspring liver, and to alter ozone-induced pulmonary responses in female offspring. Furthermore, we demonstrated that a maternal fish oil diet has positive effects on *ex vivo* polarization of offspring CD4⁺ T cells, but also adverse effects on maternal and offspring health. Lastly, we showed that maternal fatty acid status during pregnancy is associated with offspring inflammation and development of inflammatory lung conditions. These findings aid to better understand how maternal nutrition affects offspring health and may contribute to improve the health of future generations.

Impact paragraph

Objective and findings

The prevalence of obesity has nearly doubled since 1980 [1], resulting in 52% of the European Union's (EU's) adult population being overweight and 16% being obese [2]. At the same time, the prevalence of asthma has increased over the past few decades [3], leading to a current asthma prevalence of 5.4% in the EU [4]. Furthermore, respiratory infections are currently the fourth leading cause of both death and burden of disease in the world [5]. Next to their detrimental effects on a patient's health status and quality of life, these conditions have one thing in common: their development is associated with the fat content or fatty acid composition of the maternal diet during pregnancy. Given the increasing prevalences of these metabolic, immunologic and pulmonary conditions, and considering the substantial health care costs that are associated with treating them, society may benefit from preventing these diseases by improving maternal nutrition during pregnancy.

Although the long-term phenotypic effects in offspring of the maternal dietary fat content or fatty acid composition have been studied extensively, the molecular responses and mechanisms that are involved in these effects remain largely unknown. Therefore, this thesis aimed to study the effects of maternal dietary fatty acids and high-fat diet on the molecular responses related to offspring immunologic, metabolic and pulmonary health. In **chapter 2** we showed that a perinatal high-fat diet induced oxidative stress, and altered DNA methylation and gene expression of pathways involved in lipid metabolism, oxidative stress response and cell proliferation in offspring liver. In **chapter 3**, a maternal high-fat diet exacerbated ozone-induced effects on pulmonary oxidant status and the molecular control of mitophagy in female offspring. In **chapter 4**, a maternal fish oil diet, which contained high amounts of n-3 polyunsaturated fatty acids (PUFAs), not only affected *ex vivo* polarization of offspring CD4⁺ T cells, but also induced anemia in both dams and offspring, and hepatic steatosis in offspring. In **chapter 5**, we used a multivariate statistical approach to demonstrate that maternal fatty acid status was associated with offspring inflammatory markers, and that maternal Mead acid levels correlated with the risk of developing lung conditions.

Scientific relevance

The findings from this thesis may contribute to scientific advancement in a number of ways. For **chapters 2 till 4**, this contribution mainly consists of the addition of fundamental knowledge about the effects of maternal nutrition on offspring health. The effects of a perinatal high-fat diet in **chapter 2** on offspring hepatic DNA methylation and gene expression were related to the known adverse effects on offspring metabolism induced by a maternal high-fat diet. These findings may suggest that the effects of maternal diet on

offspring health are transmitted via epigenetics and the regulation of gene expression. Similarly, the effects of a perinatal high-fat diet in **chapter 2** on oxidative stress in offspring liver and in **chapter 3** on ozone-induced oxidative stress in offspring lung suggest that oxidative stress may play an important role in the effects of maternal high-fat diet on offspring health. The effect of a maternal fish oil diet in **chapter 4** on *ex vivo* polarization of offspring CD4⁺ T cells may be involved in the anti-asthmatic effects of prenatal supplementation with n-3 PUFAs, given the importance of CD4⁺ T cell polarization in atopy development. Altogether, these findings contribute to a better understanding of the molecular responses that underlie the long-term phenotypic effects of maternal nutrition. Furthermore, these findings provide new leads for future studies to further unravel the mechanisms by which maternal exposures affect offspring health.

Chapter 5 mainly contributes to scientific advancement by demonstrating the use of a non-standard statistical approach for the examination of associations between prenatal nutrition and offspring health. In this chapter, we applied the statistical model canonical correlation analysis to study the multivariate relation between maternal fatty acid status and child inflammation. Applying this multivariate approach in future studies may aid in discovering new insights in the relation between maternal diet and offspring health.

Societal relevance

Results from this thesis, together with findings from previous and future studies, may contribute to the establishment of new dietary advises that will aid to improve the health of children that are yet to be born. By improving the immunologic, metabolic and pulmonary health, these children will have a better quality of life. Next to this, health care costs may be substantially reduced. For instance, 500 million euros are spent each year in the Netherlands to treat the consequences of obesity and indirect costs are even 2 billion euros each year [6]. For asthma, direct healthcare costs are estimated to be 287 million euros per year, with costs related to sick leave adding another 650 million euros per year [7].

Target groups

On the short term, findings from this thesis are most relevant to policy makers. (Semi-) governmental institutions such as the Research Council (Gezondheidsraad) and the Nutrition Center (Voedingscentrum) will have to collect and critically review results from our studies, as well as from related previous and future studies, and translate them into specific dietary advices. These dietary advices will then be used by healthcare professionals such as midwives, dietitians, general practitioners and gynecologists to inform and advise women who are pregnant, or plan to get pregnant, and their partners about good nutrition

before and during pregnancy. Furthermore, nutrition companies may develop food products and supplements to help women meet the requirements for good perinatal nutrition. In the end, all of this will help to improve the health, and therefore quality of life, of future generations. Society as a whole may benefit from this, through the reduction of health care costs.

Communication of results

The findings presented in this thesis have been shared with other researchers in the field through poster presentations and oral presentations at national and international conferences. Furthermore, results have been or will be published in peer-reviewed scientific journals: chapters 2 and 3 have been published, chapter 4 is under review and chapter 5 is in preparation for submission. All chapters have been or will be published under a Creative Commons license, meaning that they are openly and freely accessible to everyone. For communication to the general public, organizations like the Nutrition Center (Voedingscentrum) will have to translate findings from this thesis and from related publications into specific and understandable dietary advises.

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Samenvatting

Samenvatting

Het vetgehalte en de vetzuursamenstelling van het maternale dieet tijdens de zwangerschap en lactatie beïnvloeden de ontwikkeling van het immuunsysteem, het metabolisme en de longen van nakomelingen, zelfs tot aan volwassen leeftijd. De moleculaire mechanismes die ten grondslag liggen aan deze relatie zijn echter grotendeels onbekend. Daarom was het doel van dit proefschrift om de effecten te bestuderen van maternale vetzuren of een hoog-vetdieet op de moleculaire reacties die betrokken zijn bij de immunologische, metabole en pulmonaire gezondheid van nakomelingen.

Perinatale blootstelling aan een hoog-vetdieet leidt tot een verslechterd metabolisme in nakomelingen. Men weet echter niet goed door welke mechanismes deze langetermijneffecten worden overgebracht. We hebben daarom in muizen het effect bestudeerd van een gecombineerd paternaal en maternaal hoog-vetdieet op genexpressie en DNA methylatie in de lever van volwassen nakomelingen. In **hoofdstuk 2** hebben we aangetoond dat dit perinatale hoog-vetdieet veranderingen aanbracht in de expressie van genen die betrokken zijn bij vetmetabolisme, oxidatieve stress en celgroei. Verder veroorzaakte het perinatale dieet oxidatieve stress in de levers van nakomelingen. Daarnaast verhoogde het perinatale hoog-vetdieet de hoeveelheid DNA methylatie in bindingsplaatsen van transcriptiefactoren in het metabole gen *Lpin1*. We stellen de hypothese dat de veranderingen die we hebben waargenomen in deze studie ten grondslag liggen aan de bekende metabole verstoringen in nakomelingen ten gevolge van prenatale blootstelling aan een hoog-vetdieet.

Naast het beïnvloeden van metabolisme, kan een perinataal hoog-vetdieet ook nadelige effecten van luchtverontreiniging zoals ozon op longfunctie, -schade en -inflammatie verder verslechteren. Er wordt gesuggereerd dat deze verslechtering gerelateerd is aan disfunctioneren van mitochondriën. Het doel van **hoofdstuk 3** was daarom om in ratten te onderzoeken of een dikmakend perinataal hoog-vetdieet veranderingen kan aanbrengen in de effecten die ozon heeft op mitochondriën en de oxidantstatus van longcellen. In deze studie verhoogde acute ozonblootstelling de oxidantstatus en de moleculaire regulatie van mitofagie in de longen van nakomelingen. In vrouwelijke nakomelingen verslechterde blootstelling aan een perinataal hoog-vetdieet deze effecten, terwijl in mannelijke nakomelingen de ozoneffecten niet afhankelijk waren van het maternale dieet. Deze bevindingen suggereren dat een perinataal hoog-vetdieet invloed heeft op ozon-geïnduceerde effecten in oxidantstatus en de moleculaire regulatie van mitofagie in longen van vrouwelijke nakomelingen.

Maternale suppletie met visvetzuren tijdens de zwangerschap is geassocieerd met een vermindering van astmaklachten. Ook hiervan zijn de onderliggende mechanismes onduidelijk. Aangezien polarisatie van CD4⁺ T-lymfocyten een rol speelt in de ontwikkeling van astma, hebben we in **hoofdstuk 4** de effecten bestudeerd van een perinataal visvetzurendieet op de *ex vivo* polarisatie van deze cellen in muisnakomelingen. In deze studie laten we zien dat het perinatale visvetzurendieet de extracellulaire hoeveelheden van cytokines vermindert, zowel tijdens Th1- als tijdens Th2-polarisatie. Daarnaast ontdekten we dat het perinatale visvetzurendieet niet alleen anemie (bloedarmoede) veroorzaakte in zowel de moeder als de nakomeling, maar ook leververvetting induceerde in nakomelingen. Deze resultaten tonen aan dat perinatale blootstelling aan hoge hoeveelheden visvetzuren zowel positieve als negatieve kan hebben in nakomelingen. Voor toekomstige studies die de perinatale effecten van visvetzuren willen bestuderen, is het dus belangrijk dat de doseringen nauwkeurig overwogen worden.

Het is aangetoond dat de ontwikkeling van inflammatoire longaandoeningen gerelateerd is aan het maternale dieet tijdens de zwangerschap. In eerdere studies waren bepalingen van de maternale vetzuurstatus en statistische analyses echter vaak suboptimaal. Daarom hebben we in **hoofdstuk 5** de relatie tussen maternale vetzuurstatus en inflammatie in het kind op zevenjarige leeftijd bestudeerd door data van het MEFAB cohort te analyseren met behulp van canonical correlation analysis (CCA), een multivariaat statistisch model. In deze studie laten we zien dat maternale vetzuurstatus geassocieerd is met inflammatie in het kind op zevenjarige leeftijd en dat het vetzuur Meadzuur (C20:3n-9) een belangrijke bijdrage had in deze associatie. Verder waren maternale Meadzuur-plasmaspiegels op de dag van geboorte significant gecorreleerd met de kans op het ontwikkelen van inflammatoire longaandoeningen in het kind. Toekomstige studies moeten uitwijzen of het belang van Meadzuur is toe te wijzen aan het feit dat Meadzuur een biomarker is voor een tekort aan essentiële vetzuren, of aan de eigen pro-inflammatoire functies van Meadzuur, of mogelijk aan een combinatie van beiden.

Samengevat had dit proefschrift als doel om de effecten van maternale vetzuren en maternaal hoog-vetdieet te bestuderen op de moleculaire mechanismes gerelateerd aan de immunologische, metabole en pulmonaire gezondheid van de nakomeling. We hebben aangetoond dat een maternaal hoog-vetdieet in staat is genexpressie en DNA methylatie in de lever van nakomelingen aan te passen en daarnaast ook ozoneffecten op de long kan beïnvloeden in vrouwelijke nakomelingen. Verder hebben we aangetoond dat een maternaal visvetzurendieet van de ene kant een positief effect heeft op de *ex vivo* polarisatie van CD4⁺ T cellen, maar daarnaast ook negatieve effecten heeft op de gezondheid van zowel moeder als nakomelingen. Als laatste hebben we getoond dat maternale vetzuurstatus tijdens de zwangerschap geassocieerd is met inflammatie en de

ontwikkeling van inflammatoire longaandoeningen in nakomelingen op latere leeftijd. Deze bevinden dragen bij aan meer kennis over hoe maternale voeding de gezondheid van nakomelingen beïnvloedt en kunnen mogelijk helpen om de gezondheid van toekomstige generaties te verbeteren.

About the author

About the author

Sven Rouschop was born on June 23rd 1992 in Maastricht, The Netherlands. He went to secondary school at Trevianum Scholengroep in Sittard, where he finished the Gymnasium in 2010. For his scientific education, Sven studied Molecular Life Sciences at Wageningen University. During the Bachelor's program, he did a minor in Health and Medical Biology and performed his internship at the department of Virology, where he studied cap-snatching of Schmallenberg virus in mosquito and mammalian cells. During the Master's program, he further specialized in Biomedical Research and performed two internships. For the first internship, Sven studied the effects of tastants on gut hormone secretion and their underlying signaling pathways at the Department of Nutrition and Pharmacology. The second internship was performed at MSD Animal Health, where he studied expression and purification of recombinant proteins for vaccine production. After graduating from university, Sven started his PhD research in 2016 at the department of Pharmacology and Toxicology at Maastricht University. During this PhD project, he studied the molecular effects of maternal dietary fat content and fatty acids on the immunologic, metabolic and pulmonary health in offspring. In 2017, Sven was awarded the KNAW Ter Meulen Beurs, which allowed him to visit the Risch lab at Salzburg University. In addition to performing research, Sven followed several courses related to statistics, animals research and clinical research, and visited (inter)national conferences, where he gave oral presentations and won two poster prizes. Furthermore, he was involved in teaching, for which he coordinated and supervised practical courses, supervised interns and guided problem-based learning groups. After finishing his PhD, Sven will continue his career at Hezelburcht Grant Consultancy.

List of publications

List of publications

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